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14. ABSTRACT

The study of breast cancer tumorigenesis and metastasis depends on the use of established breast cancer cell lines, which don't accurately represent the heterogeneity and complexity of human breast tumors. We developed a tumor model using primary breast tumor-initiating cells isolated from patient core biopsies which would more accurately reflect human breast cancer and its metastasis. Tumorspheres were successfully isolated from all patient core biopsies, independent of the ER/PR/Her2 status or grade. Tumorspheres demonstrated a cell surface expression phenotype CD44+/CD24low/ESA+ and were shown to secrete MMP-2 and MMP-9 in vitro. Injection of ≤503 cells in combination with matrigel into the mammary fat pad of NUDE mice resulted in the formation of small, palpable tumors, which were sustained up to 12 months post-injection. Micrometastasis was detected 3 months post-injection of tumorspheres into the mammary fat pad by PCR for human chromosome 17 in the lung, liver, kidneys, brain and femur. Macrometastatic lesions were detected as early as 6 months post-injection of tumorspheres into the mammary fat pad in the lung, liver and kidneys. Despite aberrant E-cadherin expression in the primary tumors, re-expression of E-cadherin was observed in the membrane of metastatic cells in close proximation to hepatocytes in the liver. Additionally, re-expression of E-cadherin was observed in the membrane of the majority of metastatic cells in the lung.

15. SUBJECT TERMS
Metastasis, dormancy, primary breast tumor-initiating cells, mouse models

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I. Introduction:

Breast cancer is a heterogeneous disease that remains the second leading cause of death among women. Metastatic disease increases mortality from breast cancer by 70% and is the leading cause of death in breast cancer patients independent of the manageability of the primary disease. Although generally correlated with later stages in disease progression, there is mounting evidence suggesting the metastatic process may initiate earlier in breast cancer development. Therefore, tumor volume and diagnosis may not accurately predict the presence of metastatic disease or the initiation of the metastatic process. Efficacious treatments for metastatic disease depends on development of preclinical tumor models that better predict patient response, increase understanding of the metastatic process, and enable the identification of biomarkers for early and accurate detection of metastasis.

The study of breast cancer has depended heavily upon the use of established breast cancer cell lines, whose origin is often from pleural effusions or metastatic lesions. Although significant advancements have been made possible through the use of established cell lines, further progress depends on the development of tumor models that more accurately represent the heterogeneous nature of human breast tumors. Hetero-transplantation of primary tumor biopsies from patients into immune-deficient mice has many advantages over standard xenografts from cancer cell lines. The hetero-transplant tumors can be directly compared to the original patient tumor biopsies, and to annotated information on patient features, family history, patient outcome etc. A study of breast cancer hetero-transplants revealed that patients whose breast cancer biopsies grew as tumors in mice predicted a worse prognosis compared to biopsies that did not grow tumors [1]. Unfortunately, only a very small percentage of human breast tumor tissue directly transplanted into immune-deficient mice results in tumor formation [1-3]. The identification of breast cancer stem cells (bCSCs) in breast tumors shifted the previously held hypothesis that all cells within a tumor retained the ability to recapitulate the tumor [4]. bCSCs, present in tumors at very low frequency [5], have been implicated in breast tumor progression [6], metastasis [7] and recurrence [8]. The relative quiescence of bCSCs [9] and the elevated expression of ABC transporter family of proteins [10] may contribute to bCSCs evasion of traditional chemotherapy and radiotherapy. Furthermore, recent data has shown that chemotherapeutics [11,12] and radiation [13] may enrich for bCSCs, possibly increasing risk of recurrence. Expression of ER (estrogen receptor) and Her2/neu in bCSCs remains unclear, suggesting that ER and HER2/neu directed drugs may be ineffective in targeting bCSCs [14].

A subset of cells isolated from primary breast tumors are termed breast tumor-initiating cells (bTICs) for the ability to form tumors upon injection of low numbers into the mammary fat pad of immune-deficient mice [5]. bTICs consist of a heterogeneous population of cells that include a small percentage of bCSCs as well as a range of less to more differentiated progenitor cells. bTICs have been shown to exist *in vitro* as tumorspheres upon selection under non-

adherent, serum-free conditions [15]. Recently, it has been suggested that bTICs are the cells within tumors with metastatic potential and the ability to "seed" in distant organs [16-18]. Therefore, the challenges in targeting bTICs likely extend from the primary site of tumor formation to distant metastatic sites as well. Given the evidence that supports bTICs as the cells with metastatic potential and the source of breast cancer recurrence, tumor models that employ bTICs isolated directly from patient biopsies may provide a more reliable means for study of the metastatic process and tumor recurrence. Therefore, the purpose of this study is to develop a novel and reproducible breast cancer heterotransplant model using bTICs isolated as tumorspheres from patient biopsies for the investigation of the metastatic process.

II. Research Accomplishments Body:

Aim 1. Characterize tumorspheres isolated from patient core biopsies *in vitro* and *in vivo*

Rationale: Breast-tumor initiating cells (bTICs) were first isolated from human breast carcinoma based on the surface expression profile $CD44^{+}/CD24^{low}/ESA^{+}$ using fluorescence-activated cell sorting (FACS) [4]. Subcutaneous injection of low numbers of the sorted cell population resulted in the formation of tumors comprised of a heterogeneous population of cells, reflective of the composition of the primary human tumor from which the cells were originally isolated [4]. Following the identification of bTICs, an *in vitro* method for the isolation of bTICs from human breast cancer lesions as tumorspheres was demonstrated by Ponti et al [15]. These reports, describing the identification and isolation of bTICS from human cancer lesions, prompted the feasibility of a tumor model which would more accurately recapitulate the etiology of human breast cancer and metastasis. Therefore, Specific Aim 1 will develop an optimal protocol for the isolation and propagation of tumorspheres from patient core biopsies. Upon isolation, the cell surface expression profile of the cells comprising the tumorspheres will be characterized and their tumorigenicity upon injection of low numbers of cells into the mammary fat pad of mice will be determined. Matrix metalloproteinases (MMPs) secreted by breast cancer cells have been demonstrated to promote tumor invasion and metastasis [19-21]. Therefore, the secretion of MMPs by the cells comprising the isolated tumorspheres *in vitro* will be determined to provide initial support for the hypothesized metastatic potential expected *in vivo*.

Optimize of a protocol for the efficient and reproducible isolation and propagation of tumorspheres from patient core biopsies

Results: Tumorspheres were successfully isolated from patient core biopsies under serum-free, non-adherent culture conditions. The majority of patient samples were Grade 2 or higher and diagnosed as invasive ductal carcinoma (IDC), however the ER/PR/Her2 status of the samples was variable (see Table 1). Tumorspheres isolated from patient samples ranged in size from 30 μm to 100 μm and were estimated to comprise of 50-100 cells (Figure 1A), based on cell counts

performed during the tumorsphere formation efficiency assay described below. Tumorsphere formation efficiency (TFE) experiments, performed to determine the ability of the cells within the tumorspheres to form new tumorspheres *in vitro* after enzymatic dissociation, proved unsuccessful (data not shown). Dissociation of the tumorspheres using proteolytic enzyme combinations resulted in generation of new tumorspheres *in vitro,* however subsequent passages beyond the first passage/dissociation resulted in decreased tumorsphere formation due to apparent global cell death. The addition of conditioned media from tumorspheres did not alter the ability of the dissociated cells to generate new tumorspheres or improve cell viability (data not shown). As a result of the unsuccessful attempts to propagate the tumorspheres *in vitro*, most of the experiments outlined in the statement of work were not able to be completed. However, we will report significant findings concerning the metastatic phenotype discovered upon propagation of the cells in a mouse model. The mouse model to be described below would be the first model using primary breast tumor-initiating cells which would permit the study of dormancy of disseminated breast cancer cells *in vivo*.

Determine the cell surface marker phenotype of the cells comprising the isolated tumorspheres

Results: ICC demonstrated a $CD44^{+}/CD24^{low\text{-}med}/ESA^{+}$ cell surface marker phenotype of the cells comprising the tumorspheres with no signal detected in the negative controls (Figure 1B-C). These results demonstrate the presence of breast tumor-initiating cells within the tumorspheres isolated from patient core biopsies under specified culture conditions, without the use of additional selection processes.

Determine the secretion of matrix metalloproteinases (MMPs) by tumorspheres *in vitro*

Results: MMP-1 and TIMP-1 and -2 were detected in the conditioned media of sample 6 and MMP-1, -3, -7 and -13 in addition to TIMP-1 and -2 were detected in the conditioned media of sample 7 using the ExcelArray Human MMP/TIMP array (Figure 2A). MMP-2 and -9 were detected in the conditioned media of sample 7, whereas only MMP-9 was detected in the conditioned media of sample 6 using the ExcelArray MMP/TIMP array. The ExcelArray was used as an initial screen for the presence of MMPs/TIMPs in the conditioned media of tumorspheres *in vitro.* Tumorspheres isolated from Sample 5 were not in culture, and therefore conditioned media could not be collected for the ExcelArray experiment. Biopsies from Sample 8 and 9 had not been received yet. Therefore, only conditioned media from Sample 6 and Sample 7 was analyzed for the presence of MMPs/TIMPs by the ExcelArray. However, gelatin zymography was used to confirm the results obtained from the protein array performed on the conditioned media from samples 6 and 7, as well as detect the secretion of active MMP-2 and MMP-9 by tumorspheres isolated from Samples 5-9 (Figure 2B). Active and latent MMP-2 and MMP-9 were detected in the conditioned media of samples 5-9 (data not shown). The secretion of active MMP-2 and MMP-9 by cells comprising the tumorspheres suggests a potentially invasive phenotype *in vitro* [22].

Determine the tumorigenicity of the cells comprising the tumorspheres and characterize the resultant tumors

Results: Injection of Matrigel alone into the mammary fat pad of NUDE mice did not result in tumor formation (Figure 3A). Injection of 50 tumorspheres (estimated total cells injected: 1-5x10^3 cells) in combination with Matrigel into the mammary fat pad resulted in palpable tumor within 3 months post-injection, which were maintained until the end of the experiment (approx. 9-12 months post-injection) (Figures 3B-C). These tumors were serially transplantable through NUDE mice upon re-isolation of tumorspheres *in vitro* and re-injection of tumorspheres into the mammary fat pad of mice. H+E staining showed that the edges of the tumors were occupied by a dense population of cells as compared to areas closer to the center of the tumor that were less dense (Figures 4A). IHC using a rabbit monoclonal ki67 antibody (Figures 4B, D) and terminal deoxynucleotidyl transferase (TdT)- mediated dUTP nick end labeling (TUNEL) (Figures 4C, E) demonstrated equivalent rates of proliferation and apoptosis. All tumor samples were negative for the estrogen receptor α and vimentin (data not shown). Fibronectin expression was detected in all tumor samples (Figures 5C, F). Additionally, all samples demonstrated expression of a range of cytokeratins as detected by a broad spectrum cytokeratin antibody (data not shown). E-cadherin, which is normally expressed in the membrane of epithelial cells, was observed at variable levels in both the cytoplasm and in the nucleus, but not the membrane in all tumor samples (Figures 4A, F). β-catenin, which is normally bound to the cytoplasmic tail of E-cahderin at the membrane, is a In support of the aberrant expression of E-cadherin, cytoplasmic and nuclear localization of β-catenin was also observed at variable levels in all the tumor samples (Figures 4B, F). ALDH1A1 demonstrated less than 20% of cells in all tumors expressed ALDH1A1 and no expression was detected in tumors formed from sample 8 (Figure 5F). IHC for cytokeratin 8 and cytokeratin 14 was performed to determine the presence of luminal and myoepithelial cell lineages, respectively, within the tumors. Cytokeratin 8 was detected in all samples at variable rates between tumors (Figures 5E, F) whereas cytokeratin 14 was only detected in tumors formed from samples 5 and 9 (Figure 5F). To confirm that tumors contained cells of human origin, IHC was performed using a mouse monoclonal antibody against human nuclear antigen (HNA). Cells within all the tumor samples were positive for HNA (data not shown).

Aim 2. Determine the metastatic potential of the cells comprising the tumorspheres

Rationale: Disseminated breast cancer cells may be present at distant sites at the time of primary diagnosis of breast cancer in patients that exhibit no outward signs of clinical metastasis. Current models of breast cancer metastasis have provided great insight into the contributing molecular mechanisms, however fail to recapitulate the dormancy period observed clinically. Exit from the dormant state is necessary for the development of macro-metastatic lesions in distant organs, yet the mechanisms involved are poorly understood [16,23,24]. Recent reports

have implicated breast cancer stem cells in metastasis, given their innate dormant state and ability to proliferate and differentiate upon activation [11,16]. Tumorspheres isolated in Specific Aim 1 are expected to contain a small subset of bCSCs. Active MMPs were shown to be secreted by the cells comprising the isolated tumorspheres *in* suggesting a possible invasive phenotype. Additionally, the aberrant expression of E-cadherin and cytoplasmic/nuclear localization of β-catenin further support the expected metastatic phenotype *in vivo*. Therefore, it is hypothesized that the cells comprising the tumorspheres injected into the mammary fat pad will have the potential to metastasize to various organs.

Identify and quantify the organs with disseminated human cancer cells

Results: Micrometastasis to mouse kidney, liver, lung, brain and femurs (bone marrow) was assessed at three months post-injection. No visual macrometastatic lesions were observed within any of the organs at three months. Human DNA was detected in the kidneys, liver, lung, bone marrow, and brain using PCR for human chromosome 17 (Figure 6A). As a negative control, DNA was isolated from the organs of a mouse injected with Matrigel alone into the mammary fat pad; no signal was detected (Figure 6B). H+E staining revealed metastatic lesions in the lung, liver, brain and kidneys (Figures 6C-F; Figures 7D-F). The percentage of all organs with metastasis (without regard for the size of the metastatic lesions) was comparable between samples (Figure 8A). Sample 7 demonstrated the lowest overall average (71%) and Sample 9 demonstrated the highest overall average (100%). Analysis of the number of organs with metastasis (without regard for the size of the metastatic lesions) revealed qualitative differences in the organ tropisms of the samples (Figure 8B). Samples 5 and 9 appear to have the highest tropism to lung as compared to the other samples. Samples 5 and 9 additionally demonstrated the largest metastatic burden (% metastatic burden) in the lung, indicating tropism to the lung as well as possible enhanced survival and proliferation as compared to the other samples analyzed (Figures 9A and C respectively). Although Sample 9 demonstrated the highest tropism to liver, the metastatic burden in the liver did not exceed 10%. In contrast, Sample 7 showed the lowest tropism to liver (Figure 8B) but demonstrated the highest metastatic burden in the liver as compared to the other samples (and as compared to the other organs analyzed within Sample 7) (Figure 9B). Samples 6 and 8 didn't demonstrate tropism to any particular organ and the metastatic burden was comparable between organs (data not shown). There was no apparent correlation between time (days post-injection) and metastatic burden (Figures 10A-E). However, Sample 7 appears to demonstrate the development of larger metastatic lesions at earlier time points as compared to the other samples (Figure 10C).

Characterize the disseminated cancer cells present in the organs

Results: Preliminary results have demonstrated E-cadherin was expression of E-cadherin in the membrane within the cells in the metastatic lung lesion (Figure 11G). E-cadherin was expressed

in the membrane and cytoplasm in a select subset of cells in the lesion in the liver (Figure 11F). The majority of the cells within the lesions in the liver and lung were positive for HNA (Figure 11D, E respectively). Experiments to confirm the preliminary results and determine the expression levels of estrogen receptor and β-catenin are to be completed.

Aim 3. Determine whether the disseminated cells in the bone marrow are tumorigenic and/or metastatic upon re-injection into the mammary fat pad

Rationale: Recent reports have presented data supporting the hypothesized bi-directional flow of disseminated cells, demonstrating targeted homing of disseminated cells to tumors present in the mammary fat pad and accelerated tumor progression upon colonization [25,26]. Disseminated breast cancer cells in the bone are known to exist in a dormant state for extended periods of time, maintaining the ability to proliferate upon activation to form a detectable metastatic lesion [18,27-29]. Evidence of the bi-directional flow of metastatic cells combined with the likely presence of undetectable disseminated cancer cells in a large proportion of breast cancer patients (even in cases of early detection) further complicates our understanding of recurrence at the primary site of tumor formation and the development of metastatic disease. The tumorigenicity of the disseminated cells present in the bone has yet to be determined. The detection of micrometastasis in the bone marrow of mice injected with tumorspheres permits the investigation into the tumorigenicity and metastatic behavior of the tumor cells present in the bone marrow upon injection of total bone marrow into the mammary fat pad.

Determine the tumorigenicity of disseminated human cancer cells in the bone marrow

Results: To determine the tumorigenicity of the disseminated breast cancer cells in the bone marrow, bone marrow was flushed from the femurs of mice previously injected with tumorspheres into the mammary fat pad. The flushed bone marrow was washed in PBS then combined with Matrigel Basement membrane matrix and injected into the third mammary fat pad. Bone marrow was flushed from non-injected age-matched mice and injected into the mammary fat pad to as a negative control. Tumors formed in the mammary fat pad upon injection of isolated bone marrow from femurs of mice previously injected with tumorspheres in the mammary fat pad (Figures 12C-E). Injection of total bone marrow isolated from non-injected mice did not result in any tumor formation (Figure 12B). The time to tumor formation and the size of the tumors varied between injections. The factors influencing size of the tumors formed and time to tumor formation remain in question. Cell number injected, passage number of the tumorspheres injected into the mammary fat pad of the mice from which the bone marrow was isolated, and time between injection of tumorspheres and isolation of bone marrow could variables affecting tumor formation.

Characterize the tumors formed in the mammary fat pad and compare/contrast to the tumors formed upon injection of tumorspheres from Aim 1.

Results: Experiments to be completed.

Identify and quantify the organs with disseminated human cancer cells

Results: The percentage of all organs with metastasis (without regard for the size of the metastatic lesions) was comparable between Samples 5 BM (bone marrow transplantation experiment using bone marrow isolated from mice previously injected with Sample 5 tumorspheres) and Sample 7 BM. Additionally, the percentage of all organs with metastasis was comparable between tumorsphere injection experiments (Samples 5 and 7) and bone marrow transplantation experiments (Sample 5 BM and Sample 7 BM) (Figure 13A). Whereas Sample 5 BM showed a decreased tropism to the lung as compared to Sample 5, Sample 7 BM demonstrated an increased tropism to the lung as compared to Sample 7 (Figure 13B). Sample 5 BM and Sample 7 BM demonstrated a decreased tropism to the brain as compared to Sample 5 and 7, respectively (Figure 13B). In conjunction with the indicated changes in tropism, a decrease in metastatic burden was detected in the lungs isolated from Sample 5 BM experiments as compared to Sample 5 experiments (Figure 14A). Furthermore, an increase in metastatic burden was detected in lungs in Sample 7 BM experiments as compared to Sample 7 experiments (Figure 14B). No correlation between time (days post-injection) and metastatic burden was detected. However, the bone marrow transplantation experiments indicate an earlier development of detectable metastatic lesions in comparison to the tumorsphere injection experiments (Figure 15 A-B).

4. Characterize the disseminated cancer cells present in the organs

Results: Experiments to be completed.

III. Key Research Accomplishments

Aim 1. Characterize tumorspheres isolated from patient core biopsies *in vitro* and *in vivo*

Months 1-12

- Isolation of breast tumor-initiating cells from patient core biopsies as tumorspheres *in vitro*
- $CD44^{+}/CD24^{low}/ESA^{+}$ cell surface phenotype of cells comprising tumorspheres
- Cells comprising tumorspheres secrete active MMP-2 and MMP-9 *in vitro*
- Cells comprising the tumorspheres are tumorigenic upon injection of low numbers of cells into the mammary fat pad of NUDE mice
- Tumors formed upon injection of tumorspheres are small, heterogeneous lesions
- Equivalent rates of proliferation (ki67) and apoptosis (TUNEL) is highly suggestive the lesions are in a state of tumor dormancy

Aim 2. Determine the metastatic potential of the cells comprising the tumorspheres

Months 1-12

- Disseminated cancer cells detected in the lung, liver, kidney, brain and femurs of mice injected with tumorspheres into the mammary fat pad 3 months post-injection
- After a significant lag time (~5 months), visible metastatic lesions detected in the lung, liver, and kidney
- All 5 samples have comparable metastatic potential, however organ tropism between the samples varies
- Organ tropism did not predict or correlate with the development of macrometastatic lesions within samples
- The size of the metastatic lesions did not show any correlation with time post-injection

Determine the tumorigenicity of disseminated human cancer cells in the bone marrow

Months 1-12

- Micrometastatic lesions detected in the liver, lung, kidneys, brain and femur 3 months post-injection by PCR and H+E staining
- Macrometastatic lesions detected 8-12 months post-injection in the liver, lung and kidney
- Heterogeneity between samples in tropism to particular organs as well as size of the metastatic lesions
- Indication of organ tropism did not predict or correlate with the metastatic burden present in those organs
- Selective re-expression of E-cadherin within the metastatic cells evident between the organs

Determine whether the disseminated cells in the bone marrow are tumorigenic and/or metastatic upon re-injection into the mammary fat pad

- Disseminated cells in the bone marrow (isolated from mice previously injected with tumorspheres into the mammary fat pad) are tumorigenic upon re-injection into the mammary fat pad
- Cells are metastatic to the lung, liver, kidney and brain; percentages were comparable to percent metastasis in the lung, liver, kidney and brain upon injection the primary tumorspheres

IV Reportable Outcomes

Manuscripts:

Marsden CG, Wright MJ, Pochampally R, Rowan BG. 2009. Breast tumor-initiating cells isolated from patient core biopsies for study of hormone action. *Methods Mol Biol.* 590:363-75.

Abstracts/Posters:

- **Marsden C,** Wright MJ, Carrier L, Krzysztof K, Pochampally R, Rowan B. 2011. "Primary Breast Cancer Cells With Metastatic Potential Isolated From Human Invasive Ductal Carcinoma" DOD Era of Hope Conference.
- **Marsden C,** Wright MJ, Carrier L, Krzysztof K, Pochampally R, Rowan B. 2011. "Primary Breast Cancer Cells With Metastatic Potential Isolated From Human Invasive Ductal Carcinoma" Tulane Health Sciences Research Days.
- **Marsden C,** Wright MJ, Carrier L, Pochampally R, Rowan B. 2010. "Isolation of Tumor Initiating Cells with Metastatic Potential from Human Primary Invasive Ductal Carcinoma" Tulane Health Sciences Research Days.
- **Marsden C,** Wright MJ, Carrier L, Pochampally R, Rowan B. 2009. "Isolation of Tumor Initiating Cells with Metastatic Potential from Human Primary Invasive Ductal Carcinoma" CTRC-AACR Annual San Antonio Breast Cancer Symposium.
- **Marsden C,** Wright MJ, Carrier L, Pochampally R, Rowan B. 2009. "Isolation of Tumor Initiating Cells with Metastatic Potential from Human Primary Invasive Ductal Carcinoma" e.Hormone.
- **Marsden C**, Wright MJ, Carrier L, Pochampally R, Rowan B. 2009. "Isolation of Tumor Initiating Cells with Metastatic Potential from Human Primary Invasive Ductal Carcinoma" Louisiana Cancer Research Consortium Annual Retreat.
- **Marsden C**, Wright MJ, Carrier L, Pochampally R, Rowan B. 2009. "Isolation of Tumor Initiating Cells with Metastatic Potential from Human Primary Invasive Ductal Carcinoma" Tulane Health Sciences Research Days.

V. Conclusion

Although breast cancer is diagnosed in about 10% of women between the ages of 40-50, Nielsen et al. detected subclinical lesions in 39% of women who died of trauma [30,31]. Microscopic lesions can remain asymptomatic for extended periods of time, with a high percentage never progressing to clinical manifestation [32,33]. Despite its important clinical implications in recurrence and metastatic latency, the mechanisms contributing to breast cancer dormancy remain poorly understood [34-36]. Current understanding of progression from dormancy to malignancy is significantly hindered by the lack of dormant tumor models that accurately recapitulate human breast cancer latency. Herein, we have developed a model that recapitulates human breast cancer metastasis, demonstrating a latency period with the potential for eventual malignancy. Primary breast tumor-initiating cells can be isolated as tumorspheres

under non-adherent, serum free culture conditions from patient core biopsies independent of assigned grade or ER/PR/Her2 status. Isolated tumorspheres were tumorigenic in NUDE mice and had the capacity to metastasize from the primary site (i.e. mammary fat pad) to distant organs, such as the liver, lung, kidney, brain, and femur. Tumor cells at the metastatic sites exhibited organ-specific phenotypes that demonstrated plasticity of the metastatic cells dependent upon the organ microenvironment. This model will provide researchers an indispensable tool for the study of breast cancer metastasis and dormancy.

Reference List

1. Giovanella BC, Vardeman DM, Williams LJ, Taylor DJ, De Ipolyi PD, Greeff PJ *et al*.: **Heterotransplantation of human breast carcinomas in nude mice. Correlation between successful heterotransplants, poor prognosis and amplification of the HER-2/neu oncogene.** *Int J Cancer* 1991, **47:** 66-71.

2. Mattern J, Bak M, Hahn EW, Volm M: **Human tumor xenografts as model for drug testing.** *Cancer Metastasis Rev* 1988, **7:** 263-284.

3. Marangoni E, Vincent-Salomon A, Auger N, Degeorges A, Assayag F, de CP *et al*.: **A new model of patient tumor-derived breast cancer xenografts for preclinical assays.** *Clin Cancer Res* 2007, **13:** 3989-3998.

4. Al Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF: **Prospective identification of tumorigenic breast cancer cells.** *Proc Natl Acad Sci U S A* 2003, **100:** 3983-3988.

5. Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL *et al*.: **Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells.** *Cancer Res* 2006, **66:** 9339-9344.

6. Dalerba P, Cho RW, Clarke MF: **Cancer stem cells: models and concepts.** *Annu Rev Med* 2007, **58:** 267-284.

7. Wicha MS: **Cancer stem cells and metastasis: lethal seeds.** *Clin Cancer Res* 2006, **12:** 5606-5607.

8. Al Hajj M: **Cancer stem cells and oncology therapeutics.** *Curr Opin Oncol* 2007, **19:** 61-64.

9. Wicha MS, Liu S, Dontu G: **Cancer stem cells: an old idea--a paradigm shift.** *Cancer Res* 2006, **66:** 1883-1890.

10. Hadnagy A, Gaboury L, Beaulieu R, Balicki D: **SP analysis may be used to identify cancer stem cell populations.** *Exp Cell Res* 2006, **312:** 3701-3710.

11. Yu F, Yao H, Zhu P, Zhang X, Pan Q, Gong C *et al*.: **let-7 regulates self renewal and tumorigenicity of breast cancer cells.** *Cell* 2007, **131:** 1109-1123.

12. Li HZ, Yi TB, Wu ZY: **Suspension culture combined with chemotherapeutic agents for sorting of breast cancer stem cells.** *BMC Cancer* 2008, **8:** 135.

13. Phillips TM, McBride WH, Pajonk F: **The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation.** *J Natl Cancer Inst* 2006, **%20;98:** 1777-1785.

14. O'Brien CS, Howell SJ, Farnie G, Clarke RB: **Resistance to endocrine therapy: are breast cancer stem cells the culprits?** *J Mammary Gland Biol Neoplasia* 2009, **14:** 45-54.

15. Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D *et al*.: **Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties.** *Cancer Res* 2005, **65:** 5506-5511.

16. Li F, Tiede B, Massague J, Kang Y: **Beyond tumorigenesis: cancer stem cells in metastasis.** *Cell Res* 2007, **17:** 3-14.

17. Charafe-Jauffret E, Ginestier C, Iovino F, Wicinski J, Cervera N, Finetti P *et al*.: **Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature.** *Cancer Res* 2009, **69:** 1302-1313.

18. Balic M, Lin H, Young L, Hawes D, Giuliano A, McNamara G *et al*.: **Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype.** *Clin Cancer Res* 2006, **12:** 5615-5621.

19. Duffy MJ, Maguire TM, Hill A, McDermott E, O'Higgins N: **Metalloproteinases: role in breast carcinogenesis, invasion and metastasis.** *Breast Cancer Res* 2000, **2:** 252-257.

20. Wilson TJ, Singh RK: **Proteases as modulators of tumor-stromal interaction: primary tumors to bone metastases.** *Biochim Biophys Acta* 2008, **1785:** 85-95.

21. Noel A, Hajitou A, L'Hoir C, Maquoi E, Baramova E, Lewalle JM *et al*.: **Inhibition of stromal matrix metalloproteases: effects on breast-tumor promotion by fibroblasts.** *Int J Cancer* 1998, **76:** 267-273.

22. Hagemann T, Robinson SC, Schulz M, Trumper L, Balkwill FR, Binder C: **Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF-alpha dependent up-regulation of matrix metalloproteases.** *Carcinogenesis* 2004, **25:** 1543-1549.

23. Fidler IJ: **The organ microenvironment and cancer metastasis.** *Differentiation* 2002, **70:** 498-505.

24. Barkan D, Green JE, Chambers AF: **Extracellular matrix: a gatekeeper in the transition from dormancy to metastatic growth.** *Eur J Cancer* 2010, **46:** 1181-1188.

25. Kim MY, Oskarsson T, Acharyya S, Nguyen DX, Zhang XH, Norton L *et al*.: **Tumor self-seeding by circulating cancer cells.** *Cell* 2009, **139:** 1315-1326.

26. Leung CT, Brugge JS: **Tumor self-seeding: bidirectional flow of tumor cells.** *Cell* 2009, **139:** 1226-1228.

27. Bussard KM, Gay CV, Mastro AM: **The bone microenvironment in metastasis; what is special about bone?** *Cancer Metastasis Rev* 2008, **27:** 41-55.

28. Welch DR, Cooper CR, Hurst DR, Lynch CC, Martin MD, Vaidya KS *et al*.: **Metastasis Research Society-American Association For Cancer Research Joint Conference on Metastasis.** *Cancer Res* 2008, **68:** 9578-9582.

29. Barkan D, Kleinman H, Simmons JL, Asmussen H, Kamaraju AK, Hoenorhoff MJ *et al*.: **Inhibition of metastatic outgrowth from single dormant tumor cells by targeting the cytoskeleton.** *Cancer Res* 2008, **68:** 6241-6250.

30. Nielsen M, Thomsen JL, Primdahl S, Dyreborg U, Andersen JA: **Breast cancer and atypia among young and middle-aged women: a study of 110 medicolegal autopsies.** *Br J Cancer* 1987, **56:** 814-819.

31. Black WC, Welch HG: **Advances in diagnostic imaging and overestimations of disease prevalence and the benefits of therapy.** *N Engl J Med* 1993, **328:** 1237-1243.

32. Folkman J, Kalluri R: **Cancer without disease.** *Nature* 2004, **427:** 787.

33. Indraccolo S, Favaro E, Amadori A: **Dormant tumors awaken by a short-term angiogenic burst: the spike hypothesis.** *Cell Cycle* 2006, **5:** 1751-1755.

34. Brackstone M, Townson JL, Chambers AF: **Tumour dormancy in breast cancer: an update.** *Breast Cancer Res* 2007, **9:** 208.

35. Holmgren L, O'Reilly MS, Folkman J: **Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression.** *Nat Med* 1995, **1:** 149-153.

36. Almog N: **Molecular mechanisms underlying tumor dormancy.** *Cancer Lett* 2010, **294:** 139-146.

Table 1. Breast tumor-initiating cells isolated from human breast core biopsies injected into the mammary fat pad of NUDE mice

Sample	Formation in mice	Passage in Mice	Metastasis	Age	Diagnosis	Grade	ER/PR/Her2 Status
4	0/2	N/A	Not Determined	56yrs	IDC	Grade 2	$ER^+/PR^+/Her2^-$
5	5/6	Yes	Yes	44yrs	IDC	Grade 3	$ER^-/PR^-/Her2^-$
6	6/6	Yes	Yes	62yrs	IDC with lymphovascular invasion	Grade 2	$ER^+/PR^+/Her2+$
7	5/6	Yes	Yes	77yrs	IDC	Grade 2	$ER^-/PR^-/Her2^-$
8	4/6	Yes	Yes	63yrs	IDC	Grade 2	$ER^+/PR^+/Her2?$
9	6/6	Yes	Yes	66yrs	IDC	Grade 1	$ER^+/PR^+/Her2+$

Table 1. Formation of primary tumor and metastasis in NUDE mice implanted with tumorspheres isolated from human breast core biopsies. Tumor formation in NUDE mice following bilateral injections into the mammary fat pad of tumorspheres that were derived from the original patient biopsy. 'Passage in mice' indicates that the primary tumor from above could be serially transplanted into mice to form subsequent primary tumors following *in vitro* formation of tumorspheres prior to injection into the mammary fat pad. Metastasis was determined by detection of human chromosome 17 by real time RT-PCR PCR using DNA isolated from mouse organs collected from mice injected with tumorspheres into the mammary fat pad.

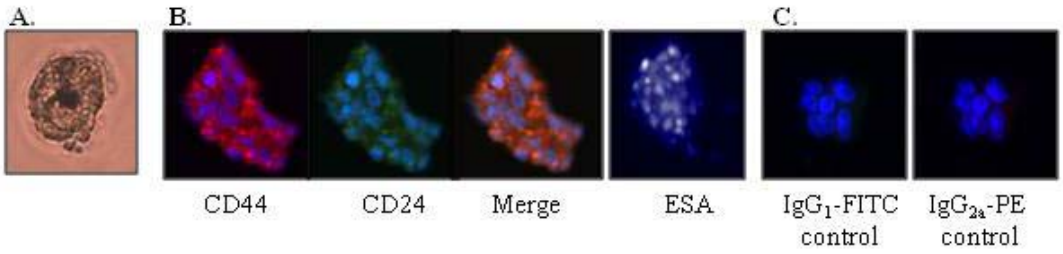


Figure 1. Characterization of tumorspheres. A. Light microscopy of a representative tumorsphere isolated from a patient core biopsy following *in vitro* culture for 10 days. **B.** Immunocytochemistry (ICC) of tumorspheres prepared by formalin fixation and 5 µm paraffin-embedded sections using pre-conjugated antibodies against CD44-PE, and CD24-FITC. ICC for ESA-FITC was performed on tumorspheres prepared by centrifugation onto glass coverslips (cytospins). Tumorspheres demonstrate a $CD44^+/ CD24^{low}/ESA^+$ cell surface marker phenotype. **C.** Isotype matched, pre-conjugated IgG control antibody mixture (IgG_1-PE/IgG_{2a}-FITC) was used as a negative control for ICC. 200x magnification in all panels.

A.

SAMPLE	MMP-2	MMP-9
SAMPLE 6	1.5 ng/ml	<0.1ng/ml
SAMPLE 7	5.5 ng/ml	0.6 ng/ml

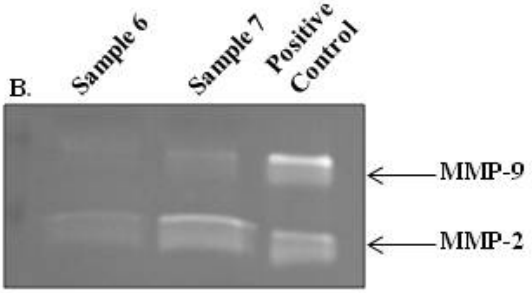


Figure 2. Secretion of matrix metalloproteinases by tumorspheres *in vitro.* **A.** The ExcelArray Human MMP/TIMP Array was used for the quantitative analysis of MMP-2 and MMP-9 in the conditioned medium of tumorspheres cultured *in* vitro. Conditioned media was collected from tumorspheres (Samples 6 and 7) following 9 days in culture. **B.** Gelatin zymography was performed using conditioned media collected from tumorspheres following 9 days in culture. The presence of active MMP-2, and MMP-9 is indicated by the arrows. Conditioned medium from 3 x10^5 A549 cells (adenocarcinomic human alveolar basal epithelial cells) stimulated with 1 ng/ml recombinant TGF-β1 for 48 hr was used as a positive control.

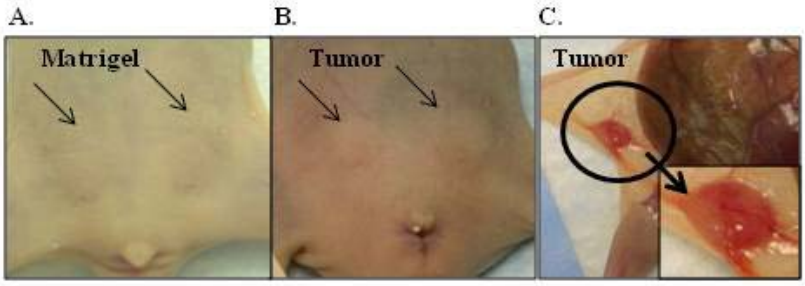


Figure 3. Tumor formation in the mammary fat pad upon injection of human tumorspheres **A.** Injection of Matrigel alone into the 3rd mammary fat pad of female NUDE mice did not result in tumor formation at up to 8 months post-injection. **B, C.** ≤ 5x10^3 cells derived from the original patient biopsy were injected into the 3rd mammary fat pad in the form of 'tumorspheres' (with Matrigel) and resulted in formation of small, palpable tumors by 3 months post-injection with an approximate, sustainable tumor volume of 100mm^3.

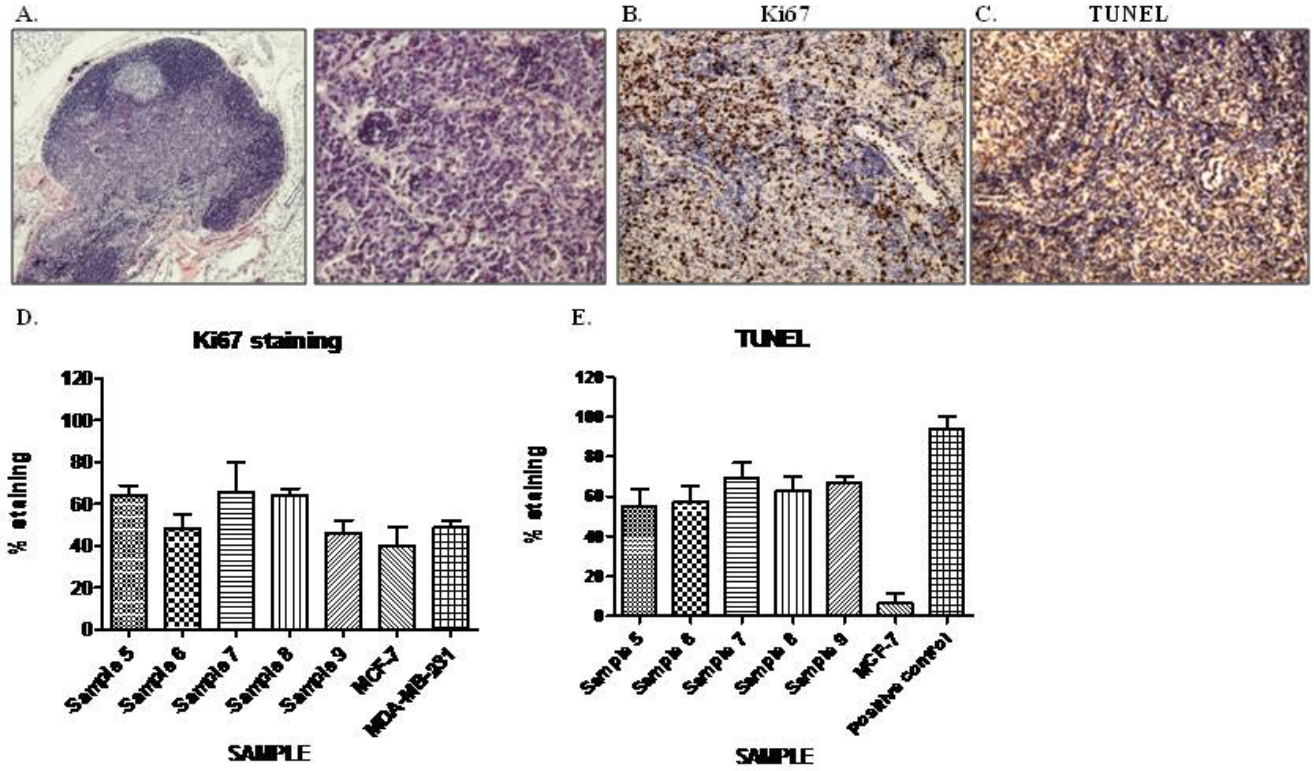
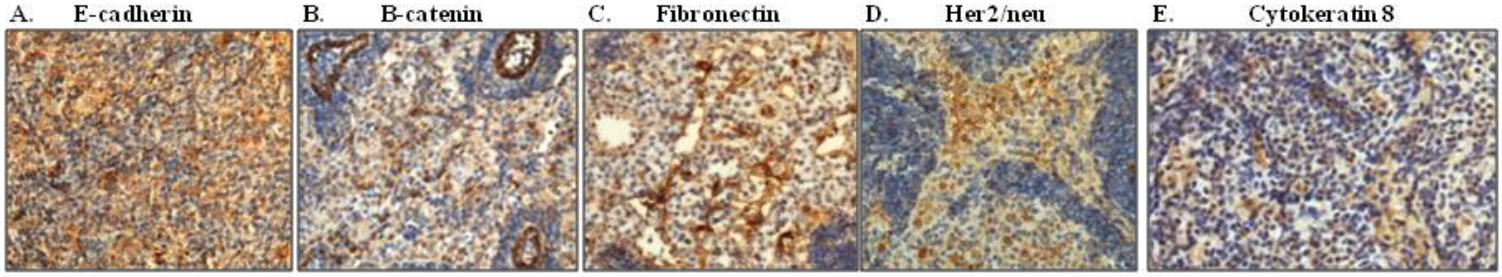


Figure 4. Basic characterization of primary tumors formed upon injection of tumorspheres. A. Hematoxylin/eosin staining of 5 µm paraffin-embedded tumor sections derived from patient Sample 5 at 40x and 200x, respectively. **B-E**. IHC for Ki67 (pre-diluted, rabbit anti-human monoclonal ki67 antibody, Thermo scientific) **(B+C)** and TUNEL (*in situ* cell death detection kit, POD, Roche) **(C+E)** were performed on 5 µm paraffin-embedded tumor sections derived from patients Samples 4-9. MDA-MB-231 human breast cancer xenograft incubated with 4 U/ml DNase I (NEB) was used as a positive control for TUNEL. Values are reported as mean +/- SD.



F.

SAMPLE	E-cadherin	β-catenin	Fibronectin	Her2/neu	Cytokeratin 8	ALDH1A1	Cytokeratin 14
5	38.4%+/-15.6	58.4%+/-12.3	42.2%+/-6.50	58.1%+/-13.2	65.4%+/-12.3	13.6%+/-4.67	42.4%+/-10.8
6	59.7%+/-18.9	66.0%+/-13.2	58.6%+/-12.37	39.4%+/-16.0	29.7%+/-4.9	13.1%+/-6.95	Negative
7	69.5%+/-8.3	76.4%+/-4.2	71.1%+/-17.1	28.0%+/-11.2	40.5%+/-6.5	15.0%+/-6.1	Negative
8	80.5%+/-5.7	61.3%+/-12.8	78.8%+/-7.8	72.3%+/-6.0	66.7%+/-10.8	Negative	Negative
9	53.9%+/-7.7	52.3%+/-9.4	61.3%+/-9.6	45.9%+/-16.1	49.1%+/-13.3	19.1+/-8.3	19.6%+/-10.4

Figure 5. Immunohistochemistry indicates molecular heterogeneity between tumor samples and within tumor samples. A-F. Representative micrographs demonstrating patterns of staining of E-cadherin (rabbit monoclonal antibody, Cell Signaling), β-catenin (rabbit polyclonal antibody, Cell Signaling), Fibronectin (rabbit polyclonal antibody, Abcam), Her2.neu (rabbit monoclonal antibody, Cell signaling), and cytokeratin 8 (rabbit polyclonal antibody, Abcam) respectively in tumors formed upon the injection tumorspheres into the mammary fat pad of female NUDE mice**.** 200x magnification in all panels. **F.** Histoscores applied to each sample for each antibody using the particle count command in the ImageJ 1.42I program.

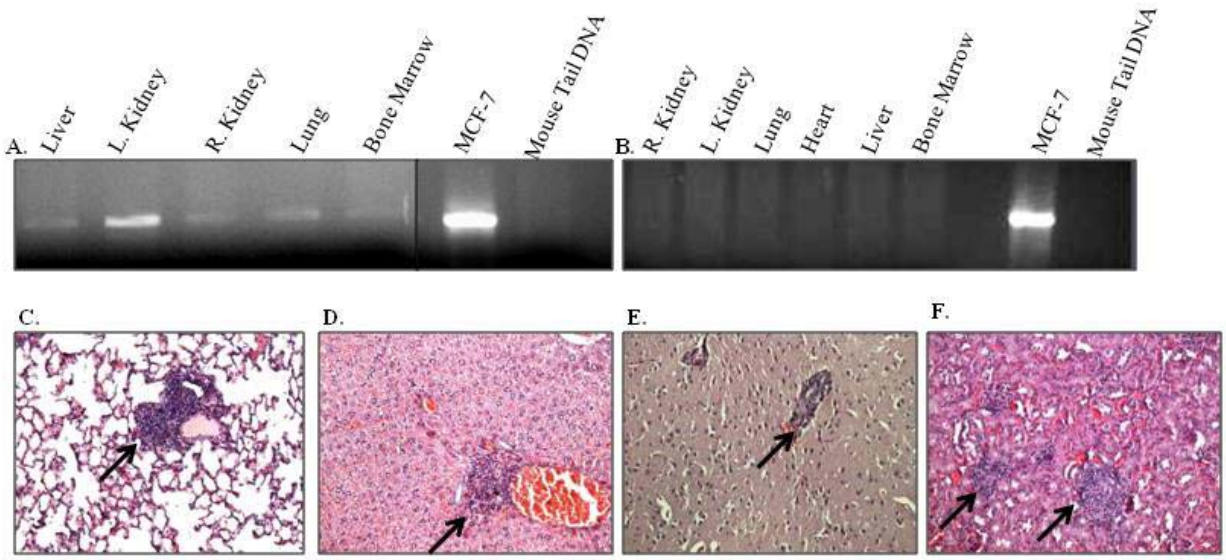


Figure 6. Detection of micrometastatic human cancer cells in mouse tissues. **A**. PCR for a centromeric region in human chromosome 17 was used to detect human cells in mouse organs isolated from mice injected with tumorspheres into the mammary fat pad three months post-injection. Human DNA was detected in the lungs, kidneys, brain, bone marrow, and liver. DNA isolated from MCF-7 cells and the mouse tail was used as a positive and negative control, respectively. **B**. PCR for DNA isolated from organs collected from a mouse injected with Matrigel alone was also used as a negative control. **C-F.** Micrographs representing micrometastasis in the lung, liver, brain and kidney, respectively, isolated from mice injected with tumorspheres into the mammary fat pad. 100x magnification in all panels.

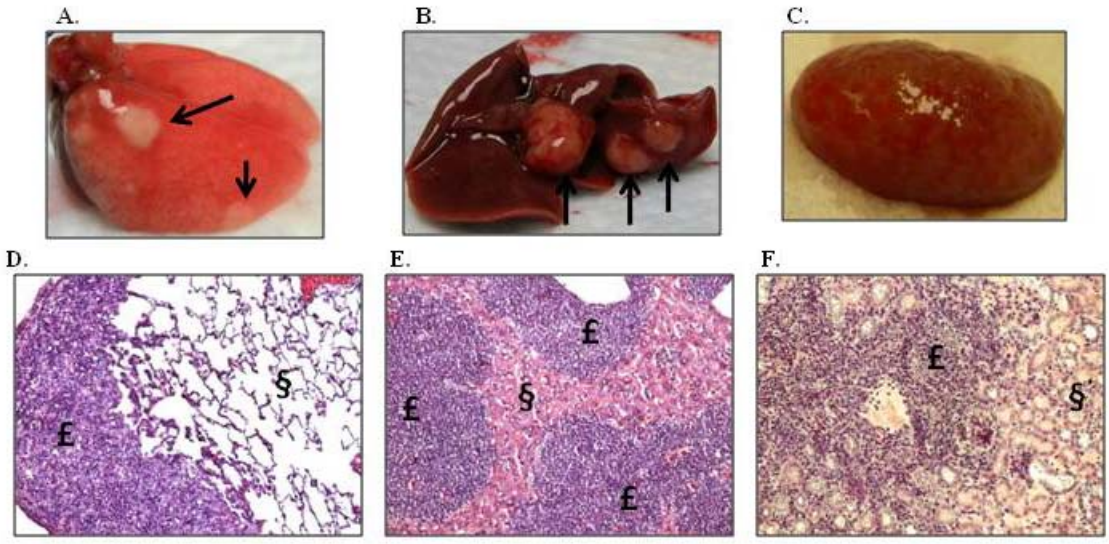


Figure 7. Macrometastatic lesions in the organs of mice injected with tumorspheres into the mammary fat pad. **A-C.** Representative visual metastatic lesions detected in the lung, liver, and kidney, respectively, 10 months post-injection of tumorspheres into the mammary fat pad of NUDE mice. **D-F.** Hematoxylin and eosin staining performed on 5µm paraffin-embedded section of a lung, liver and kidney, respectively, illustrates metastatic lesions in the organs. Lesion indicated by £; normal tissue indicated by §. 100x magnification in all panels.

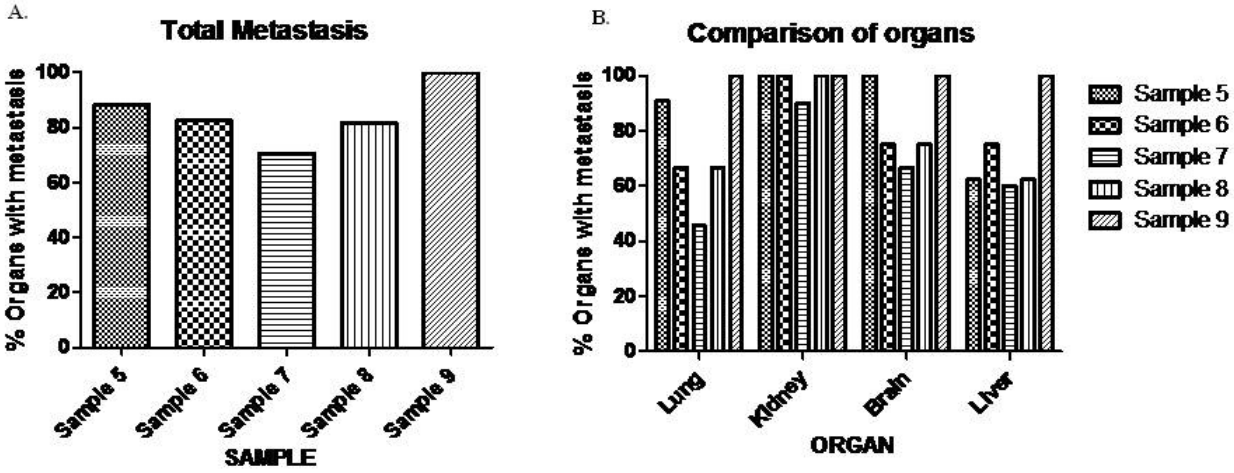


Figure 8. Quantification of the organ tropism of the metastatic cancer cells. A. Comparison of the percentage of total organs analyzed with metastatic cells, determined by H+E staining of 5µm paraffin-embedded sections of the lungs, kidneys, brain and liver, between samples 5-9. **B.** Comparison of the percent of lungs, kidneys, brains and livers analyzed with metastatic cells, determined by H+E staining of 5µm paraffin-embedded sections of the lungs, kidneys, brain and liver, between the samples 5-9.

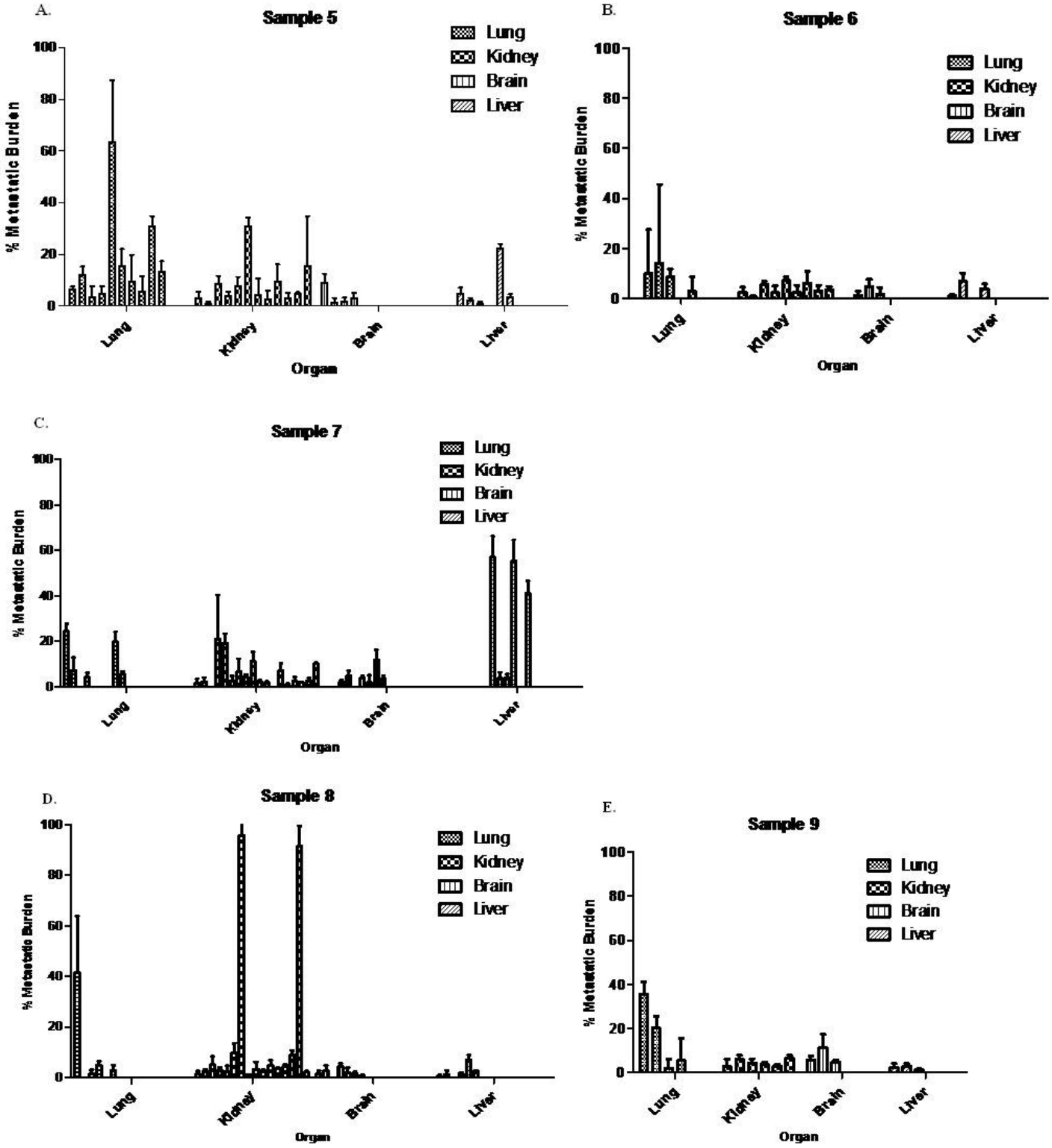


Figure 9. Quantification of the metastatic burden within the mouse organs. A-C. Graphical representation of the metastatic burden determined for each lung, kidney, brain and liver analyzed for Samples 5-9. The metastatic burden in each organ was calculated by dividing the pixels present in the metastatic lesion/s by the total pixels comprising the field of view then multiplying by 100 [(x pixels/y pixels)*100], resulting in a percent value. Each bar represents the average metastatic burden calculated in a single organ (lung, kidney, brain or liver) analyzed. Values are reported as mean +/- SD.

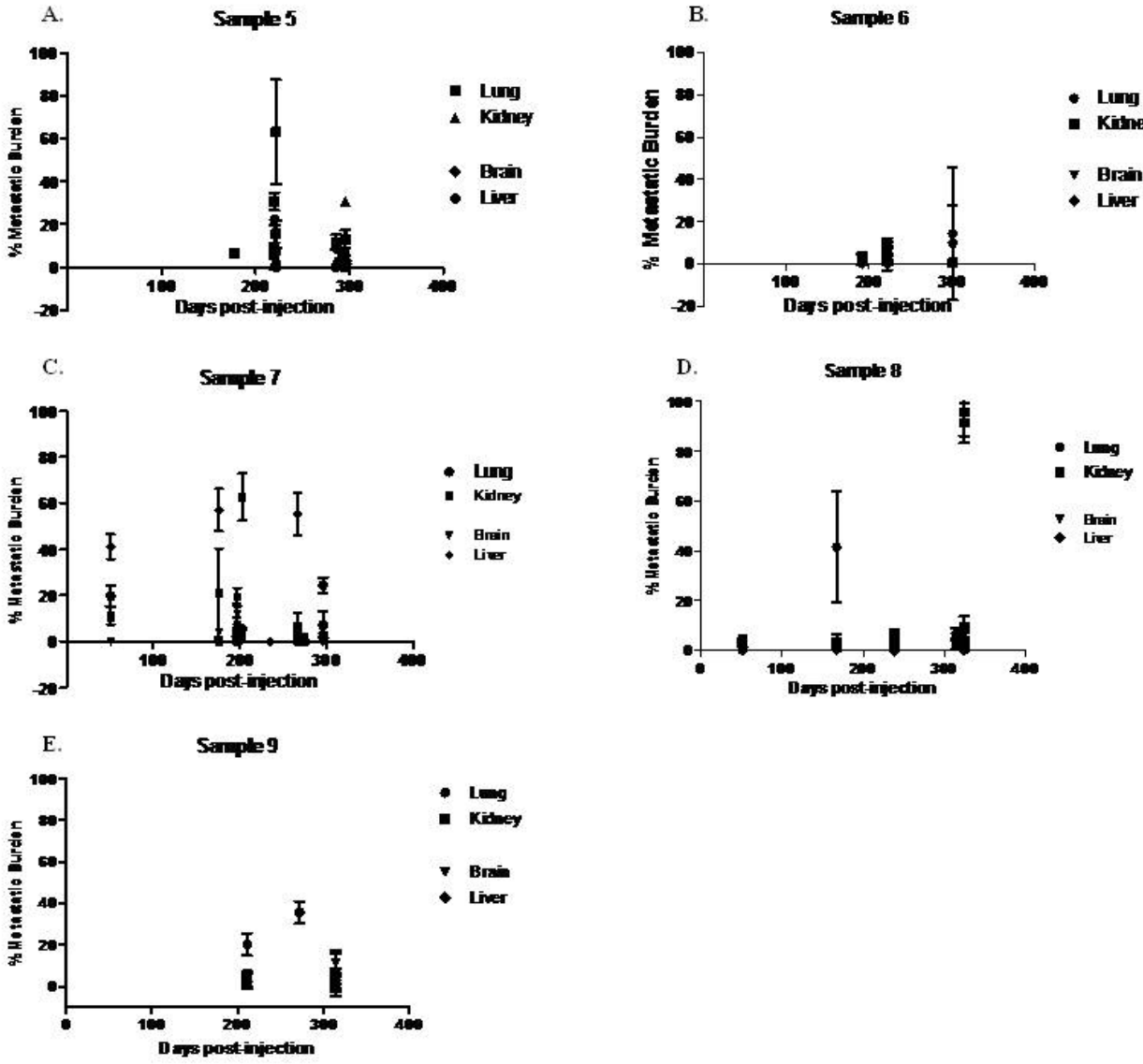


Figure 10. Correlative analysis of time post-injection and metastatic burden within the mouse organs. Graphical representation of the percent metastatic burden, previously calculated as described in Figure 9, compared to days post-injection of tumorspheres into the mammary fat pad for Samples 5-9, respectively. Values are reported as mean +/- SD.

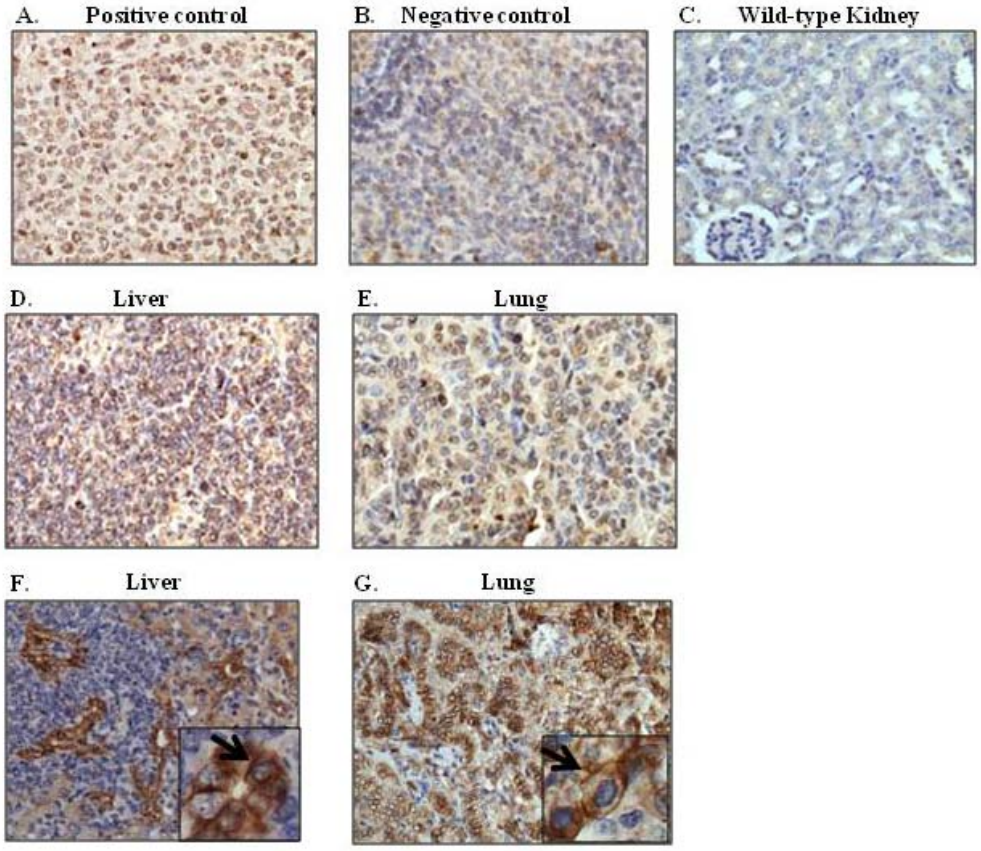


Figure 11. Characterization of the human cells at the metastatic sites. **A.** 5µm paraffin-embedded sections of a MDA-MB-231 xenograft used as a positive control for HNA (mouse anti-human nuclei monoclonal antibody) staining. **B.** Tumor sample matched negative control, with the replacement of the primary antibody with 1x PBS. **C.** Kidney isolated from a non-injected NUDE mouse, incubated with HNA to demonstrate human specificity with the lack of nuclear staining of the mouse kidney cells. **D-E.** HNA staining of 5µm paraffin-embedded sections of metastatic lesions in the liver and lung respectively confirms the human origin of the lesion, with the majority of nuclei staining positive. **F-G.** IHC performed on 5 µm paraffin-embedded sections of a liver and lung respectively using a monoclonal anti-human antibody to E-cadherin. In the metastatic lesions in the lung and the liver, E-cadherin expression was evident in the membrane and cytoplasm compared to the predominantly nuclear and cytoplasmic E-cadherin staining that was detected in the primary tumor (Figure 5A). All panels 200x magnification.

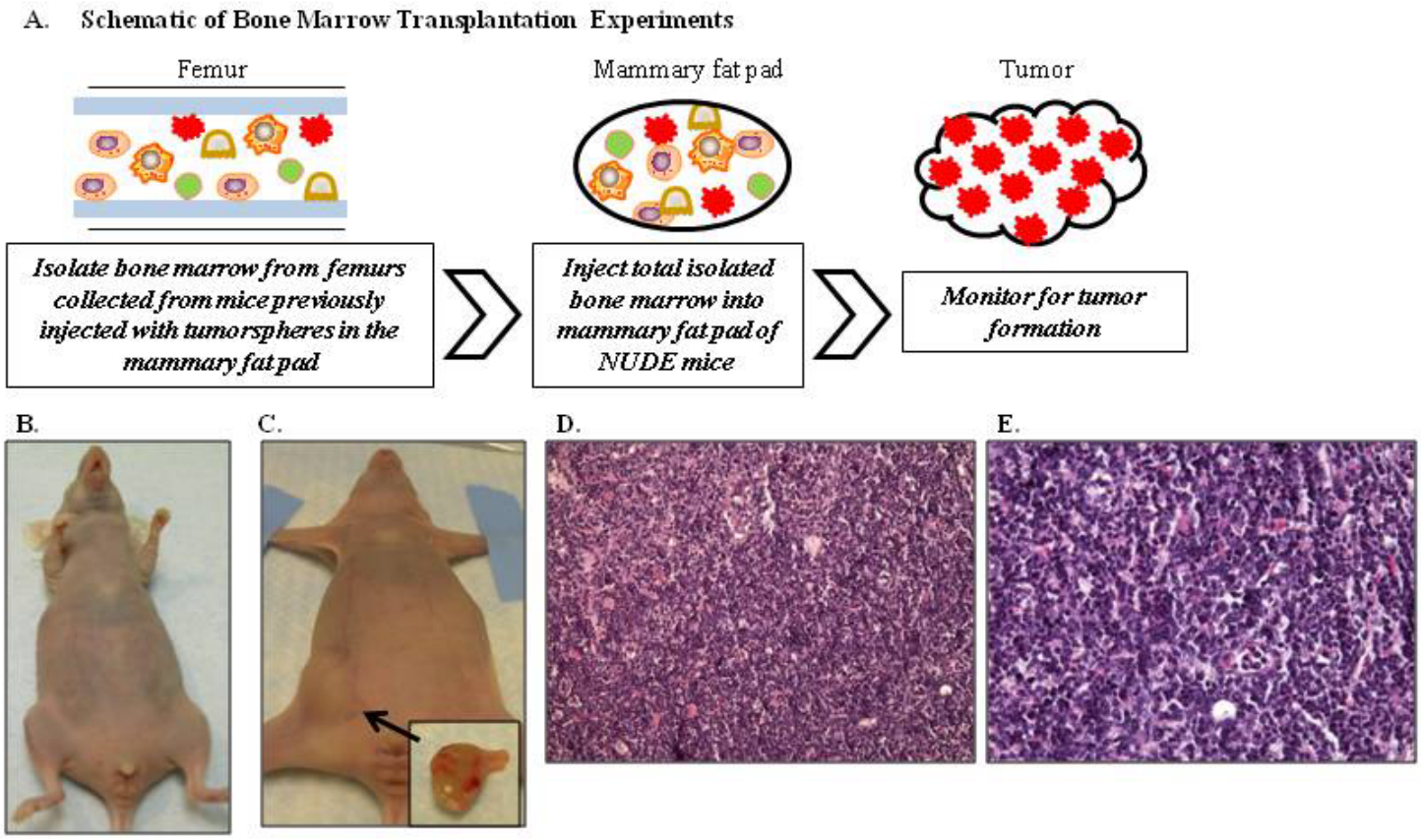


Figure 12. Tumor formation in the mammary fat pad upon injection of total bone marrow aspirate isolated from femurs of mice previously injected with tumorspheres into the mammary fat pad. A. Schematic diagram illustrating the experimental design of the bone marrow transplantation experiments. **B.** Injection of bone marrow isolated from femur of non-injected mice results in no tumor formation in the mammary fat pad. **C.** Injection of $12.5x10^6$ cells/pad aspirated from the femur of mice injected with tumorspheres 3 months prior results in large tumor formation in the mammary fat pad**. D-E.** H+E staining of 5µm paraffin-embedded tumor sections. 100x and 200x, respectively.

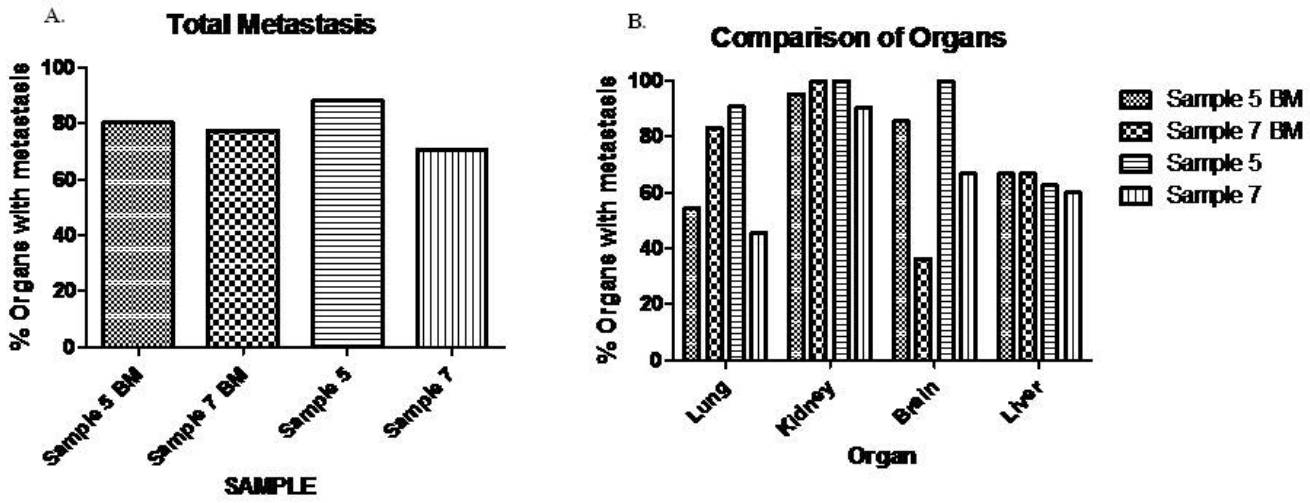


Figure 13. Quantification of the organ tropism of the metastatic cancer cells in the bone marrow transplantation experiments as compared to the tumorsphere injection experiments. Comparison of the percentage of total organs combined (lung, kidney, brain and liver) analyzed with metastatic cells, determined by H+E staining of paraffin-embedded sections of the organs, between bone marrow transplantation experiments for samples 5 and 7 (Sample 5 BM and Sample 7 BM ,respectively) and tumorsphere injection experiments for Samples 5 and 7. **B.** Comparison of the percent of lungs, kidneys, brains and livers analyzed with metastatic cells between bone marrow transplantation experiments for samples 5 and 7 (Sample 5 BM and Sample 7 BM ,respectively) and tumorsphere injection experiments for Samples 5 and 7.

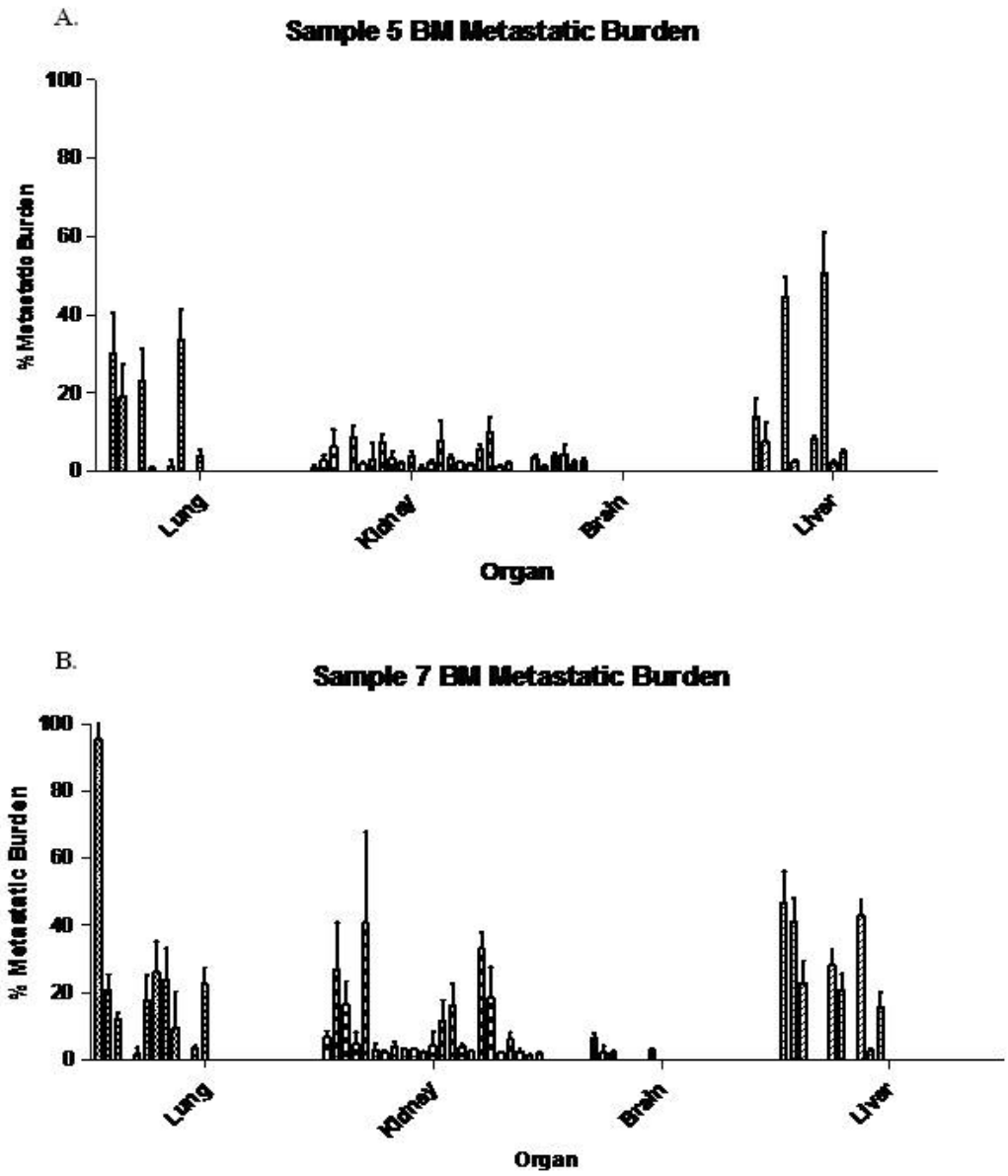


Figure 14. Quantification of the metastatic burden within the mouse organs in the bone marrow transplantation experiments. **A, B.** Graphical representation of the metastatic burden determined for each lung, kidney, brain and liver analyzed from Sample 5 BM and Sample 7 BM, respectively. The metastatic burden in each organ was calculated by dividing the pixels present in the metastatic lesion/s by the total pixels comprising the field of view then multiplying by 100 [(x pixels/y pixels)*100], resulting in a percent value. Each bar represents the average metastatic burden calculated in a single organ (lung, kidney, brain or liver) analyzed. Values are reported as mean +/- SD.

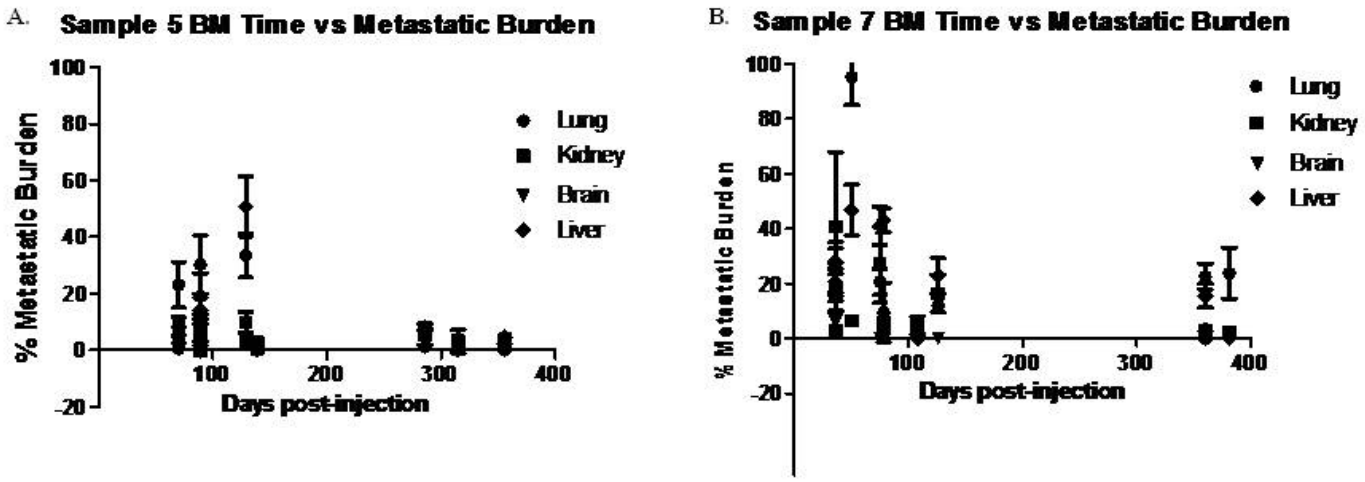


Figure 15. Correlative analysis of time post-injection and metastatic burden within the mouse organs in the bone marrow transplantation experiments. A, B. Graphical representation of the percent metastatic burden, previously calculated as described in Figure 9, compared to days post-injection of total bone marrow into the mammary fat pad. Values are reported as mean +/- SD.

Chapter 23

Breast Tumor-Initiating Cells Isolated from Patient Core Biopsies for Study of Hormone Action

Carolyn G. Marsden, Mary Jo Wright, Radhika Pochampally, and Brian G. Rowan

Abstract

In recent years, evidence has emerged supporting the hypothesis that cancer is a stem cell disease. The cancer stem cell field was led by the discovery of leukemia stem cells (Tan, B.T., Park, C.Y., Ailles, L.E., and Weissman, I.L. (2006) The cancer stem cell hypothesis: a work in progress. *Laboratory Investigation*. **86**, 1203–1207), and within the past few years cancer stem cells have been isolated from a number of solid tumor including those of breast and brain cancer among others (Al-Hajj M., Wicha M.S., Benito-Hernandez A., Morrison, S.J., and Clarke, M.F. (2003) Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. USA* **100**, 3983–3988; Singh, S.K., Clarke, I.D., Terasaki, M., Bonn, V.E., Hawkins, C., Squire, J., and Dirks, P.B. (2003) Identification of a Cancer Stem Cell in Human Brain Tumors. *Cancer Research.* **63**, 5821–5828). Cancer stem cells exhibit far different properties than established cells lines such as relative quiescence, multidrug resistance, and multipotency (Clarke, M.F., Dick, J.E., Dirks, P.B., Eaves, C.J., Jamieson, C.H.M., Jones, D.L., Visvader, J., Weissman, I.L., and Wahl, G.M. (2006) Cancer Stem Cells-Perspectives on Current Status and Future Directions: AACR Workshop on Cancer Stem Cells. *Cancer Research.* **66**, 9339–9344). In addition, our laboratory has demonstrated that breast cancer stem cells exhibit a strong metastatic phenotype when passaged in mice. Since stem cells exhibit these somewhat unique properties, it will be important for endocrinologists to evaluate hormonal action in these precursor cells for a more thorough understanding of cancer biology and development of more effective treatment modalities. A relatively easy and low cost method was developed to isolate breast cancer stem cells from primary needle biopsies taken from patients diagnosed with primary invasive ductal carcinoma during the routine care of patients with consent and IRB approval. Fresh needle biopsies (2–3 biopsies at 2 cm in length) were enzymatically dissociated in a collagenase (300 U/ml)/hyaluronidase (100 U/ml) solution followed by sequential filtration. Single cell suspensions were cultured on ultra low attachment plastic flasks in defined medium and formed non-adherent tumorspheres. The tumorspheres exhibited surface marker expression of $CD44^{+}/CD24^{low/-}/ESA^{+}$, previously defined as a "breast cancer stem cell" phenotype by Al Hajj et al. (Al-Hajj M., Wicha M.S., Benito-Hernandez A., Morrison, S.J., and Clarke, M.F. (2003) Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. USA* **100**, 3983–3988).

Key words: Breast cancer, tumor-initiating cells, primary cell culture, tumorspheres, invasive ductal carcinoma, cancer stem cells.

O.-K. Park-Sarge, T.E. Curry, Jr. (eds.), *Molecular Endocrinology,* Methods in Molecular Biology 590,
DOI 10.1007/978-1-60327-378-7_23,

1. Introduction

An understanding of the mechanisms for hormone action and endocrine therapy response in breast cancer in limited due to the complexity of this disease, the numerous disease subtypes, and de novo and acquired resistance to endocrine therapies. Part of the complexity in breast cancer is likely due to the presence of limiting numbers of cancer stem cells. By definition, cancer stem cells are tumor cells that (a) have the ability to self-renew and (b) can recapitulate the entire cellular heterogeneity of tumor from which the cells were derived when transplanted into an immunodeficient mouse model (1). In this regard, the cancer stem cell would be the progenitor of all the differentiated tumor cells that constitute the bulk of a breast tumor. Whereas the more differentiated breast tumor cells are sensitive to chemotherapy and endocrine therapy for breast cancer, the cancer stem cells are insensitive to these therapeutics by virtue of a relatively quiescent phenotype, expression of membrane pumps, and uncertain nuclear receptor status.

Tumor initiating cells (T-ICs), a term used to describe a putative stem cell population, are a population of cells that contain a sub-population of cancer stem cells (1). T-ICs have the capacity to initiate and maintain tumor growth; however the term refers to a more heterogeneous population of cells containing a population of cancer stem cells (1). Isolation of T-ICs will provide an important laboratory tool to understand endocrine regulation of these multipotent cells. This chapter describes methods for isolation, culturing, and characterization of breast T-ICs.

Breast T-ICs can be isolated using a variety of methods. Fluorescence activated cell sorting (FACS) utilizes the expression of identified cell surface markers to isolate T-ICs (2,3). The advantage of using FACS to isolate breast T-ICs is that the initial cell population will be less heterogeneous. However, propagation in vitro as tumorspheres will most likely yield a heterogeneous population of cells ranging from cancer stem cells to more differentiated progenitor cells. The low number of cells recovered from FACS is a caveat of the technique, depending on the size of the tissue and hence the total number of cells initially isolated from the primary tissue. For the isolation of breast T-ICS, an alternative to isolating T-ICs from primary tissue is FACS sorting of established breast cancer cell lines (4). This method offers accessibility and convenience due to the unlimited resource of cell lines. However, similar to the parental cell line, T-ICs sorted from cancer cell lines present translational limitations and complications from long-term culture.

The method presented in this chapter for isolating breast T-ICs is more accessible for researchers without consummate stem cell experience and cheaper than alternative methods described above.

The following method is a variation of a valid isolation method developed by Ponti et al. (5) and uses specific culture conditions to isolate non-adherent cells that form tumorspheres in vitro. One disadvantage to the following method is that a heterogeneous population of cells is isolated as compared to FACS sorting to isolate population based on specific cell surface markers. However this method provides a robust and reliable approach to isolate T-ICs for studies in which the degree of "stemness" of isolated cells is not as critical as the tumor formation efficiency and the serial transplantability. Isolated primary T-ICs provide a tumor model that more closely recapitulates the primary tumor when injected into the mammary fat pad of immunodeficient mice. For that reason, tumor models derived from T-ICs are more relevant for investigation of basic tumor biology, metastasis, and response to hormones and therapeutics.

2. Materials

2.1. Isolation of Breast T-ICs from Biopsy Core Samples

1. 1x Hanks' balanced salt solution (HBSS) with phenol red.
2. 1x Phosphate buffered solution (PBS), pH 7.0.
3. Dulbecco's Phosphate Buffered Saline (DPBS), sterile-filtered, cell culture tested (Sigma-Aldrich).
4. 7.5% bovine serum albumin (BSA) in DPBS.
5. Disposable scalpels.
6. Dulbecco's Modified Eagles Medium/Ham's F-12 (DMEM: F-12): 1:1 mix, with L-glutamine and 15 mM HEPES (#11330-032, Invitrogen).
7. 10x Collagenase (3000 U/ml)/Hyaluronidase (1000 U/ml) in Dulbecco's Modified Eagle's Medium with glucose (1000 mg D-Glucose/L) (Stem Cell Technologies).
8. Sterile Petri dishes.
9. BD Falcon Filter Cell Strainer, 100 μm and 40 μm pore size.
10. B-27 supplement (50X) (GIBCO-Invitrogen).
11. Human recombinant epidermal growth factor (EGF) is dissolved at 20 μg/ml in 10 mM acetic acid/0.1% BSA and stored in 500 μl aliquots at –20°C. The working concentration is 20 ng/ml and therefore used at a 1:1000 dilution.
12. Insulin, human recombinant (SAFC Biosciences) diluted in autoclaved ddH_2O at 400 μg/ml, using hydrochloric acid to adjust the pH to dissolve (about pH 2.7). Aliquots of 400 μg/ml stored at −20°C and used at a working concentration of 4 μg/ml.

13. Human recombinant basic fibroblast growth factor (bFGF) is dissolved at 25 μg/ml in DMEM:F-12 and stored in aliquots at –20°C. The working concentration is 10 ng/ml.
14. DMEM:F-12 complete medium. To prepare 500 ml of DMEM:F-12 complete medium, add 5 ml of 400 μg/ml of human recombinant insulin, 500 μl of 20 μg/ml human EGF, 200 μl of 25 μg/ml of bFGF, 26.7 ml of 7.5% BSA in DPBS, and 10 ml of 50X B-27 supplement to 447.6 ml of DMEM:F12. Sterile filter the final solution (*see* **Note 1**).
15. Ultra low attachment culture 75 cm^2 flasks/100 mm dishes/6-well plates/24-well plates/96-well plates (Corning), depending on the number of cells and types of experiments to be performed.

2.2. Culture Conditions and Maintenance In Vitro

1. DMEM:F-12 as in 2.1.
2. B-27 supplement (50X).
3. hEGF (20 ng/ml).
4. Insulin (4 μg/ml).
5. Human bFGF (10 ng/ml).
6. 7.5% BSA in DPBS.
7. Ultra low attachment culture 75 cm^2 flasks/100 mm dishes/6-well plates/24-well plates/96-well plates (Corning), depending on the number of cells and types of experiments to be performed.
8. 1x Trypsin with EDTA (0.05%) (Invitrogen).
9. 1x Trypsin inhibitor from *glycine max* (soybean) stored in 1 –2 ml aliquots at −20°C (Sigma-Aldrich).

2.3. Immunocytochemistry on Isolated Tumorspheres

1. 1x PBS with 2% fetal bovine serum (FBS), pH 7.4.
2. 1x PBS with 1% BSA, pH 7.4.
3. Cytocentrifuge (Cytofuge 2, Statspin).
4. Filter concentrators (FF01/FF01-B) and stainless steel clips (FFCL, Statspin).
5. Cardboard filters (supplied with concentrator and used to concentrate the cells at one spot on the slide).
6. Superfrost plus microscope slides, pre-cleaned.
7. Acetone.

2.4. Immunocytochemistry on Tumorsphere Cytospins

1. 1x PBS, pH 7.4.
2. Normal goat serum.
3. Nonidet-P40 (NP40), reagent grade.

4. Humidified chamber; an unused pipette tip box with wet paper towels and a small amount of ddH_2O in the bottom.
5. IgG1-FITC/IgG2a-PE antibody mixture (Beckman Coulter). This mixture of two nonspecific antibodies that are isotype matched for the CD44-PE antibody and CD24-FITC antibodies is used as a negative control for nonspecific staining. If using different primary antibodies then choose the appropriate isotyped matched IgG control antibody to assess nonspecific staining.
6. CD44-PE antibody and CD24-FITC antibody (BD Biosciences).
7. ESA-FITC antibody (Biomeda).
8. Hoechst 33342 dye (1 mM) to be diluted in 1x PBS, pH 7.4 to a working concentration of 10 μM.
9. Prolong Gold Antifade reagent.
10. Coverglass slips 24 × 30 mm.

3. Methods

Isolating primary breast T-ICs from patient core biopsies can be challenging. With an approved IRB protocol and patient consent, tumor biopsies may be obtained either at the time of initial patient diagnosis or following tumor resection. During initial patient diagnosis, ultrasound guided needle biopsies are efficient for obtaining core biopsies from the tumor and not the surrounding normal tissue. However all resultant cultures must be evaluated by a pathologist for cellular and nuclear morphology to determine whether cells are from cancer or normal tissue origin. There is always variability in the number of T-ICs isolated from different biopsies. Regardless of the variability, a limited number of T-ICs will be isolated from a small amount of tissue such as a core biopsy. Alternately, biopsies obtained by the surgeon following tumor resection (True-cut biopsies) may also be used as a source for T-ICs and would provide a larger amount of starting material. Once again, resection samples would have to be evaluated by a pathologist to ensure separation of cancerous tissue from normal tissue prior to isolating T-ICs. A further concern for resection samples would occur if the patient received any neoadjuvant therapy prior to resection that could introduce variability in the T-ICs. It has been demonstrated that neoadjuvant chemotherapy may actually enrich for the number of T-ICs obtained from resection

biopsies (6). Nonetheless, cultures established from resection samples should be carefully evaluated with the expectation that not all tissue samples will yield T-ICs.

Due to the possible infectious nature of the patient tissue samples, safety precautions are needed during dissociation of the tissue and during all subsequent culturing and handling of the samples. The user should consult with the appropriate safety officials for proper protection against infectious agents. Since there is no penicillin/streptomycin added to the complete medium, precise and careful cell culture techniques need to be employed. In addition, if there are established cell lines present in the same cell culture laboratory, primary cells should be used in a separate hood and designated incubator that will not contain established cell lines to minimize cross contamination.

3.1. Isolation of Breast T-ICs from Biopsy Core Samples

1. Upon receipt of core biopsies, immediately place the biopsy pieces into a 15 ml conical tube containing 1x HBSS (4°C) and place on ice for immediate transport to the laboratory.
2. Once back in the laboratory, transfer the core biopsy pieces and 1x HBSS into a sterile Petri dish in a sterile tissue culture hood.
3. Thaw 3–5 ml (depending on the amount of tissue) of 10x collagenase/hyaluronidase in a 37°C water bath. Pre-warm DMEM:F-12 in a 37°C water bath for diluting the 10x collagenase/hyaluronidase.
4. Remove the 1x HBSS from the dish and discard into a 50 ml conical tube containing bleach (*see* **Note 2**).
5. Wash the core biopsies twice with 1x PBS, pH 7.4. Removing and disposing of the 1x PBS should be done in the same manner as the 1x HBSS in 3.1.4.
6. Place the core biopsies into a new sterile Petri dish (or a sterile tissue culture plate) and add 8–10 ml of 1x PBS, pH 7.4. Using two sterile disposable scalpels mince the tumor pieces into ~2 mm pieces in 1x PBS, pH 7.4. Do not use excessive mechanical dissociation of the tissue or increased cell death will occur.
7. Transfer the minced tissue pieces in PBS to a 50 ml conical tube. Allow the tumor pieces to settle to the bottom (about 5–10 minutes).
8. While waiting, dilute the pre-thawed 10x collagenase/hyaluronidase to 1x in pre-warmed DMEM:F-12 and mix thoroughly.
9. From step 3.1.7, remove the 1x PBS supernatant without disturbing the settled minced tumor pieces and dispose the PBS in bleach as in step 3.1.4.
10. Add 30–50 ml of 1x collagenase/hyaluronidase to the minced tumor pieces and gently agitate, either by pipetting up and down or by brief and slow pulse on a vortexer (*see* **Note 3**).

11. Incubate the 50 ml conical tube containing the tumor pieces in a 37°C water bath for 3–4 hours, agitating every 15–25 minutes by pipetting up and down in the tissue culture hood. If an overnight incubation for the enzymatic digestion is preferred, additional growth factors etc. must be added to the DMEM:F-12/collagenase/hyaluronidase mixture to prevent excessive cell death. Usually, 3–4 hour enzymatic dissociation at 37°C is sufficient for enzymatic dissociation.
12. Prepare DMEM:F-12 complete medium during the enzymatic dissociation step (*see* 2.1.13).
13. Upon completion of the enzymatic dissociation, sequentially filter the cell suspension through a 100 μm pore filter, and then a 40 μm pore filter and collect the flow through into a fresh 50 ml conical tube. This step is used to remove undigested tissue and clumps of cells that will not pass through the filters and should be discarded. The flow through will contain single cells that will be used in the next step.
14. Centrifuge the resultant single cell suspension at 350g for 10 minutes. Resuspend the cell pellet in complete medium with gentle pipetting. Remove 10 μl of the resuspended cell solution for cell counting by trypan blue exclusion.
15. Plate 1000 cells/ml in an ultra low attachment plate/flask (6), depending on the total number of isolated cells in the single cell suspension. Culture cells at 37°C with 5% CO_2. Tumorspheres should be visualized by light microscopy within 3–6 days of culture. A general flow chart of the procedure and an image of a tumorsphere are shown in **Fig. 23.1**. Maintain tumorspheres under non-adherent culture conditions in the DMEM:F-12 complete media.

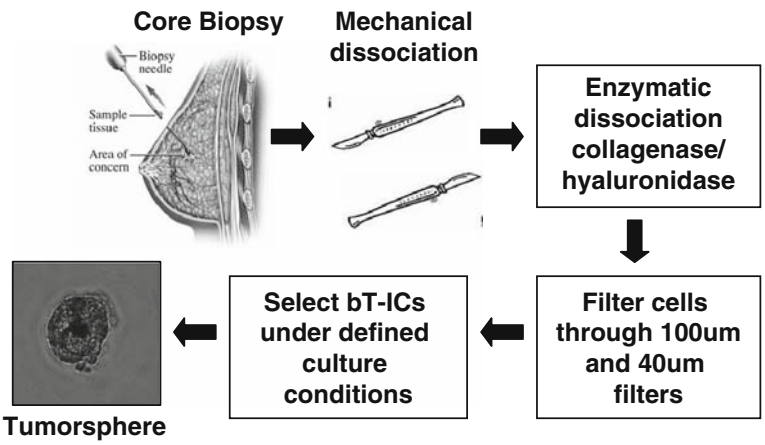


Fig. 23.1. A flow chart illustrating the general isolation procedure for bT-ICs including an image of a "tumorsphere" in vitro.bT-ICs; breast tumor initiating cells.

3.2. Culture Conditions and Maintenance In Vitro

1. When approximately 50% of the tumorspheres reach 60 μm in diameter, collect all tumorspheres in a 50 ml conical tube and centrifuge at 300*g* for 10 minutes (tumorspheres that are larger than 60 μm in diameter develop a darkened center).
2. While cells are in the centrifuge, thaw a sufficient number of aliquots of trypsin inhibitor in a 37°C water bath.
3. Carefully aspirate the supernatant (*see* **Note 4**) and gently resuspend the cells in 1 ml of 0.05% trypsin-EDTA keeping bubble formation to a minimum.
4. Incubate the 50 ml conical tube containing the cells in a 37°C water bath for 10 minutes with gentle agitation after 5 minutes.
5. After the 10 minute incubation, add an equal volume of trypsin inhibitor to the cells (e.g., if cells were incubated in 1 ml of trypsin, add 1 ml of trypsin inhibitor). Pipette the cells up and down about 40 times using a Rainin P1000 pipettor with sterile plugged pipette tips, minimizing bubble formation.
6. Centrifuge the cells at 350*g* for 10 minutes (*see* **Note 5**).
7. Gently resuspend the cell pellet in complete media with a Rainin P1000 pipettor and sterile plugged pipette tips (keeping bubble formation to a minimum). Remove 10 μl of the resuspended cell solution for cell counting by trypan blue exclusion.
8. Plate the cells at 1000 cells/ml on ultra low attachment plates/flasks in DMEM:F-12 complete medium.
9. Every 3 days, collect cells by centrifugation at 350*g* for 10 minutes and replate cells with fresh complete media containing EGF and bFGF.

3.3. Tumorsphere Cytospins

1. Harvest >100 tumorspheres in a conical tube and centrifuge at 300*g* for 10 minutes.
2. Wash (pipette up and down 3–4 times) the tumorspheres in cold 1x PBS, pH7.4 supplemented with 2% FBS twice, centrifuging at 300*g* for 10 minutes after each wash.
3. After the second wash, resuspend the tumorspheres in 100 μl of 1x PBS, pH7.4 supplemented with 1% BSA (using cotton plugged sterile pipette tips) and keep tumorspheres on ice.
4. Label the Superfrost Plus microscope slides appropriately. Be aware of the slide orientation when placing slides into the cytocentrifuge to avoid loading cells into the incorrect wells of the filter concentrators. Once assembled, it is very difficult to see the labels on the slides.
5. Assemble the filter concentrators and cardboard filters as per the manufacture instructions. Cardboard filters are used to concentrate the cells at one spot on the glass slide. Be sure that

the hole in the cardboard filter is in the proper position and the filter and the slide are flush to ensure the cells can reach the slide.

6. Place the assembled filter concentrators into the appropriate positions in the cytocentrifuge.
7. Add 100 µl of 1x PBS, pH7.4 supplemented with 1% BSA to the wells of the filter concentrators (*see* **Note 6**). Centrifuge at 500*g* for 2 minutes.
8. Quickly add 100 µl of each tumorsphere sample to the appropriate wells of the filter concentrators. Centrifuge at 500*g* for 10 minutes.
9. Remove the filter concentrator apparatus from the cytocentrifuge. Remove the metal clips being careful not to scrape the filter across the slide with the attached cells (the circle of liquid on the slide indicates the location of the cells on the slide).
10. Carefully open the filter concentrator separating the cardboard filter from the slide without any lateral motion that would disturb the attached cells on the slide.
11. Dry the slides overnight at room temperature (in a secure location to avoid any disturbance).
12. Add acetone to the slides for 5 minutes at 4°C to fix the cells.
13. Wash the slides three times with 1x PBS, pH 7.4 for 5 minutes each wash at room temperature.
14. Dry slides at room temperature but avoid excessive drying (*see* **Note 7**). Store slides at –20°C if staining won't be performed immediately.

3.4. Immunocytochemistry on Tumorsphere Cytospins

1. Blocking step: using the slides from step 3.3.14, add ~200 µl of blocking buffer (10% normal goat serum and 0.2% NP-40 in 1x PBS, pH 7.4) to slides. Incubate slides for 30 minutes in a humidified chamber at room temperature.
2. During the 30 minute blocking step, prepare primary antibody dilutions in blocking buffer (*see* step 3.4.1). For antibody incubations, at least 200 µl of diluted antibody will be needed per slide. Antibody dilutions should be determined empirically. As an example, CD44-PE, CD24-FITC, and ESA-FITC antibodies were diluted 1:100 in blocking buffer. All experiments should make use of appropriate controls (incubation of slides with isotype matched nonspecific antibody or peptide competition of primary antibody). As an example, a mixture of IgG1-FITC and IgG2a-PE (Beckman Coulter) served as isotype matched control antibodies for CD44-PE, CD24-FITC, and ESA-FITC. Control antibodies should be prepared at the same dilution in blocking buffer

(or the same mass/volume) as the primary antibodies. Some primary antibodies may be incubated concomitantly (e.g., CD44-PE and CD24-FITC antibodies may be incubated together). Keep all antibodies on ice and protected from light (*see* **Note 8**).

3. Wash the slides twice with cold 1x PBS, pH 7.4, blotting the slides on paper towels to remove the excess 1x PBS, pH 7.4 after each wash. Be sure to only touch the edge of the slide to the paper towel to avoid disturbing the attached tumorspheres.
4. Turn off as many lights in the workspace as possible before working with fluorophore-conjugated antibodies. Prepare your workspace such that exposure to light during the addition of the diluted antibodies is minimized.
5. Add ~200 µl of each antibody dilution to the appropriately labeled slides.
6. Cover the humidified chamber with aluminum foil without tipping the chamber, since this may cause loss of liquid from the desired location on the slide.
7. Incubate the slides in the humidified chamber for 90 minutes at room temperature.
8. Dilute 1 mM of Hoechst dye (*see* **Note 9**) 1:100 in 1x PBS, pH 7.4 to make a 10 µM working solution. Make enough working solution to add 500 µl to each slide. Keep working solution on ice and protect from light.
9. Remove the Prolong Gold Antifade reagent from –20°C to equilibrate to room temperature before use.
10. Wash slides twice with 1x PBS, pH 7.4 for 5 minutes each. Blot the edge of the slides on a paper towel to remove excess 1x PBS, pH 7.4 after each wash.
11. Add ~500 µl of 10 µM Hoechst 33342 dye to each slide. Incubate at room temperature for 15–20 minutes, in the humidified chamber with aluminum foil.
12. Wash slides once with 1XPBS, pH 7.4.
13. Blot the side of the slides gently on a paper towel to remove excess 1x PBS, pH 7.4.
14. Using a Pasteur pipette, make a thin line of the Prolong gold antifade reagent along one edge of the slide, adjacent to the attached tumorspheres. Pick up a coverslip using a forceps and touch the edge of the coverslip to the thin line of the Prolong gold antifade reagent. Gently drop the coverslip onto the slide. Once the Prolong gold reagent has dispersed under the coverslip, check for bubbles. If there are excess bubbles, use the forceps to gently push the bubbles to the edge of the coverslip for removal.

15. Allow the preparation to cure overnight, protected from light, before imaging. Images of the staining, including controls, are shown in **Fig. 23.2**.

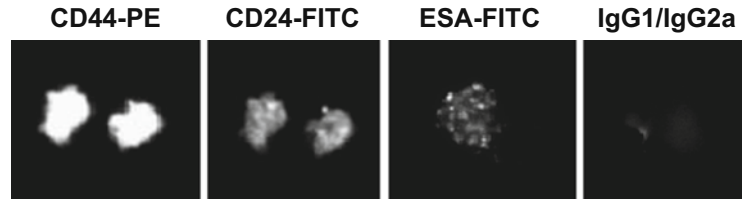


Fig. 23.2. Immunocytochemistry of stained cytospins of tumorspheres derived from breast cancer biopsy demonstrating a $CD44^{+}/CD24^{low/-}/ESA^{+}$ phenotype.

4. Notes

1. Replace the growth factors, EGF and bFGF, every 3–4 days. Store complete media for up to 1 month at 4°C, protected from light.
2. Before beginning, setup several 50 ml conical tubes containing bleach. All reagents and supplies that come into contact with the tissue pieces should be placed into the bleach before disposal.
3. For two core biopsies, typically 30 ml of 1x collagenase/hyaluronidase is sufficient for complete enzymatic digestion. For each addition core biopsy, in general, increase the amount of 1x collagenase/hyaluronidase by 10 ml.
4. Depending on the number and size (from 40 to 100 μm) of the tumorspheres in culture, a pellet may or may not be visible following centrifugation. Prior to centrifugation, mark the region of the tube where the pellet should form. If there is no pellet visible, aspirate the majority of the supernatant and then hold the tube at a 15–45° angle to the working surface of the tissue culture hood while pressing the aspirating pipette to the side of the tube to aspirate the supernatant at the bottom of the tube where the cells are pelleted (although not visible). Do not hold the tube at a sharp angle to aspirate for any length of time as the cells may detach from the bottom of the tube. In addition, do not repeatedly agitate the supernatant at the bottom of the tube while aspirating at an angle since this will detach cells from the bottom of the tube and the number of cells recovered will be significantly reduced.
5. Some protocols filter the dissociated cells through a 50–40 μm pore filter at this step to ensure a single cell suspension. We do not include this step because of cell loss during filtration. For the purpose of tumorsphere formation assays, flow

cytometry and any other assays that require a single cell suspension with the exclusion of doublets, triplets, etc. should include this filtration step. However, for the sole purpose of passaging and expanding the tumorspheres in culture, filtration is not necessary at this step.

6. If a significant number of tumorspheres were obtained and cell loss is not a concern, wetting the filter with 1x PBS, pH7.4 supplemented with 1% BSA is not necessary. Wetting the filter with 1x PBS, pH7.4 supplemented with 1% BSA before adding the tumorspheres will increase the number of cells that are deposited on the slide following centrifugation.
7. Allow slides to dry just enough to prevent crystal formation from residual PBS during storage at –20°C. Avoid excessive drying of the slide as this could decrease the efficiency and quality of subsequent staining.
8. The fluorophores conjugated to the various antibodies are photosensitive. For optimal visualization of the fluorescent signal, the antibodies should be protected from light throughout the procedure.
9. Hoechst 33342 is the preferred Hoechst dye used for this protocol. However other Hoechst dyes that are not cell permeable will also function well with this protocol since the cells were permeabilized during the fixation step with acetone and subsequent incubation with blocking buffer that contains the membrane solubilizing detergent NP-40.

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References

1. Clarke, M.F., Dick, J.E., Dirks, P.B., Eaves, C.J., Jamieson, C.H.M., Jones, D.L., Visvader, J., Weissman, I.L., and Wahl, G.M. (2006) Cancer Stem Cells-Perspectives on Current Status and Future Directions: AACR Workshop on Cancer Stem Cells. *Cancer Research.* **66**, 9339–9344.
2. Al-Hajj M., Wicha M.S., Benito-Hernandez A., Morrison, S.J., and Clarke, M.F. (2003) Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. USA* **100**, 3983–3988.
3. Yu, F., Yao, H., Zhu, P., Zhang, X., Pan, Q., Gong, C., Huang, Y., Hu, X., Su, F., Lieberman, J., and Song, E. (2007) *let-7* Regulates Self Renewal and Tumorigenicity of Breast Cancer Cells. *Cell.* **131**, 1109–1123.
4. Fillmore, C.M., and Kuperwasser, C. (2008) Human breast cancer cell lines contain stem-like cells that self-renew, give rise to

phenotypically diverse progeny and survive chemotherapy. *Breast Cancer Research.* **10**, R25.

5. Ponti, D., Costa, A., Zaffaroni, N., Pratesi, G., Petrangolini, G., Coradini, D., Pilotti, S., Pierotti, M.A., and Daidone, M.G. (2005) Isolation and *In vitro* Propagation of Tumorigenic Breast Cancer Cells with Stem/Progenitor Cell Properties. *Cancer Research.* **65**, 5506–5511.
6. Dontu, G., Abdallah, W.M., Foley, J.M., Jackson, K.W., Clarke, M.F., Kawamura, M.J., and Wicha, M.S. (2003) In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes & Development.* **17**, 1253–1270.

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- Preparation of mouse embryonic fibroblasts from mouse embryos
- Preparation of competent bacteria for common use
- Preparation of other common reagents
- Maintenance of a tissue culture facility shared between multiple laboratories
- General organization and maintenance of a laboratory, consisting of approximately 17 members, for proper functioning

2002- 2003 Boston Medical Center

Laboratory of Dr. Thomas Rothstein
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Responsibilities:

- Dialyzing and testing supernatants
- Making buffers and solutions
- Maintaining records on mouse colonies
- Ordering supplies and equipment for the laboratory
- Autoclaving materials/glassware
- Performing monthly radiation inspections and maintaining organized reports

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Cellular Biology Laboratory
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- Maintaining cotton plant population
- Glassware

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- Micro-dissections of mouse embryos (day 7.5-day 16.5)
- Preparation and maintenance of MEFs from mouse embryos
- Beta-Gal staining of Gene Trap embryos
- In situ hybridization
- Designing genotyping strategies for mouse colonies

Molecular Biology:

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- Southern Blotting
- RNA extraction/gels
- Molecular Cloning
- DNA preparation
- Electrophoresis

Cell Culture:

- Freezing/thawing cell lines
- Transfection of various cell types
- Drug selection of cell populations
- Culturing mouse embryonic stem cells
- Fluorescent microscopy
- Apoptosis assays
- Electroporation of mouse embryonic stem cells
- Differentiation of mouse embryonic stem cells

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Marsden CG, Wright MJ, Pochampally R, Rowan BG. *Breast tumor-initiating cells isolated from patient core biopsies for study of hormone action.* Methods Mol. Bio. 2009; 590: 363-75.

Liu J,Carmell MA, Rivas FV, **Marsden CG,** Thomson JM, Song J, Hammond SM, Joshua-Tor L, Hannon GJ. *Argonaute 2 is the Catalytic Engine of Mammalian RNAi*. Science. 2004 Sept 3; 305: 1437-1441.

Abstracts:

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